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FUNDAMENTALS OF BIOCHEMISTRY

FUNDAMENTALS OF BIOCHEMISTRY

in relation to Human Physiology

BY

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To Pauline

Preface to the Sixth Edition

WITH the publication of the present edition this book reaches a turning point in its history, inasmuch as for the first time it has gained an entirely new chapter. The justification for such an addition to a volume intended as an elementary survey will be found in a study of the new chapter itself, from which it will be realised that the patient work of many years has now culminated in the recognition of the sterols and their derivatives as components hardly less important than the proteins themselves in the intricate mechanism of the biochemical machine.

Apart from this the reader will discover lesser emendations at points where the earlier account needed revision.

T. R. PARSONS

CAMBRIDGE,

August, 1939.

Foreword

WHILE the study of physiological chemistry has been adequately dealt with from the *practical* point of view, the *theoretical* treatment of the subject seems still to be confined to larger treatises containing a much greater wealth of detailed information than is appropriate in an elementary introduction suitable for readers new to the subject. It appeared desirable, therefore, to attempt to describe in a continuous story the more important generally-accepted principles which have been derived from the study of the chemical changes occurring in the human body. The present volume represents the result of such an attempt. During its preparation I have borne in mind the particular needs of my own students, but, as I have demanded the minimum of previous knowledge of pure chemistry and physics, it is hoped that the book may prove not too unsuitable to a wider circle of readers.

Although the work is intended to constitute the most elementary of introductions to the subject, I have not hesitated to indicate the sources where further information can be obtained on the topics discussed. This procedure is justified, I think, even in so small a volume, for even the elementary student can gain considerable help and inspiration from the reading of only a few significant sentences from the original work of an authority, although he be unable to follow the more intricate portions of the argument. And, further, it is only when the student comes to realise that one single book can never truly suffice for the intelligent reading of any subject, but must be supplemented by the comparison and sifting of information

gleaned from as many sources as are available to him, that he can hope to make his knowledge so intimate a part of his mental equipment that it will be ready to serve him whenever he needs it, in either a professional or an academic career. The student who would *realise* his subject must turn often to the original sources of its material; he will then come to regard it not as being a kind of inborn inspiration given only to the few, but—as it really is—as being made up of the lasting results of human intellectual effort, welded into a whole which never becomes complete or flawless, but which is always being increased and perfected and ordered by the labour of men's minds. . . .

It has been my desire that any merit my book may possess may result from its containing *less* of information rather than more than other books contain. But I am fully aware that my anxiety for the omission of detail must often have run to such excess as to constitute a fault rather than a virtue. That I have not erred further in this direction is due to the kindly help I have received during my task from Professor F. G. Hopkins and Mr. S. W. Cole, to both of whom I tender my best thanks.

I wish also to thank Mr. John Murray for his courteous permission to reproduce three of the coloured diagrams of spectra from Halliburton's "Physiology."

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Autumn, 1922.

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CHAPTER I

THE NATURE OF LIVING MATTER : THE CONSTITUTION OF THE PROTEINS

“ We are such stuff
As dreams are made on, and our little life
Is rounded with a sleep.”

—*The Tempest.*

WE cannot begin our study of the chemical changes occurring in the living organism more suitably than by considering the properties and behaviour of the most important and characteristic substances that enter into the composition of all its cells and tissues—to wit, the **proteins** (Gr. $\pi\rho\omega\tau\omicron\varsigma$ = first). Of course, in the complex system that makes up a living tissue there are contained, in addition to the proteins, many other substances such as starches, sugars, fats, salts and so on, but these are of secondary importance, and represent, roughly, reserve materials waiting to be used by the living structure rather than fundamental parts of that structure itself. In other words, all that association of phenomena which we term life is manifested only by matter that is made up to a very large extent of proteins, and is never exhibited in the absence of these substances. But the investigation of the proteins has proved to be a very difficult task. In general, of course, when the chemist wishes to study the properties and constitution of any substance he endeavours first of all to prepare the substance in a pure condition—by distillation, if it be a liquid, or by oft-repeated crystallisation if it be a solid. In the case of

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the proteins, however, we have to deal with substances that are extremely liable to change. Indeed, if they did not possess this well-marked changeableness of nature they would not be suited to form the chief constituents of living tissues whose every manifestation is in essence a change—a change from rest to activity, from youth to age, from health to disease and death . . .

But it is just this liability to change which is the chief source of difficulty in the chemical study of the proteins. Take a solution of a protein, heat it but a comparatively few degrees above the normal temperature of the body, and irreversible processes of decomposition set in ; make the solution a little too acid, or too alkaline, and, once more, the original protein is broken up and lost. One has but to think of the properties of egg-white—which is composed chiefly of the protein **egg albumin**—to picture to oneself its viscid, slimy, non-crystallising solution which coagulates to an opaque white mass as a result of a relatively insignificant rise of temperature—in order to realise the serious nature of the obstacles that the properties of the typical proteins oppose to the investigation of their molecular structure. Certainly egg albumin is one of the few proteins that have been obtained in a crystalline condition, but this achievement is possible only by the employment of very accurately controlled processes of precipitation, too limited in their application to be used as routine methods of purification ; and most of the great variety of proteins that have been separated from the organs and body fluids of animals and from the tissues of plants are known only as amorphous powders. The ordinary condition of proteins in solution is not one that is adapted for the fashioning of the exquisite architecture of a crystal. There thus exist enormous

difficulties in the obtaining of most individual proteins in a condition of sufficient purity to ensure that their ultimate analysis by the accurate combustion methods employed by the organic chemist shall yield results possessing more than a rough, barely quantitative, significance. It helps us but little to know that most proteins contain about half their weight of carbon and about one-sixth of their weight of nitrogen. But, nevertheless, a very considerable amount of information with regard to the chemical constitution of the proteins has been obtained—and that mainly from a study of the products obtained by disrupting their molecules. It is a case where the structure is too intricate and too delicate for us to examine as a whole by our present clumsy methods : all we can do is to try to form some picture of the original complex by studying the fragments that remain when the destructive tendencies of our natures have been given their freedom to perform their worst upon it. The easiest way in which a protein can be broken down into simpler and more easily investigated substances is by boiling it with a dilute acid. Suppose, for example, that we take some egg-white and boil it for some hours in a reflux apparatus with dilute hydrochloric acid, then gradually the properties typical of egg-white disappear from the mixture and we are left with a solution containing the breakdown products of the egg albumin. And when we examine these products left in the solution we find that, apart from a certain amount of ammonia and small quantities of unidentified substances, the protein has been broken down completely into a mixture of substances that belong to the group of **amino-acids**. We must pause here for a moment to remind the reader exactly what an amino-acid is. He

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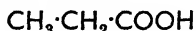
is probably quite familiar with the series of saturated fatty acids beginning with **formic acid**,



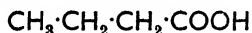
The second member of the series is **acetic acid**,



and the third, **propionic acid**,

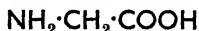


—a name which (being derived from the Gr. $\pi\rho\omega\tau\omicron\varsigma$ = first and $\pi\acute{\iota}\omega\nu$ = fat) refers to the fact that propionic acid, while resembling formic and acetic acids in being miscible with water in all proportions, differs from these two simpler members of the series by being salted out by calcium chloride and thus coming to float as an oily layer. For the present we will mention only the next member of the series, namely, **butyric acid**,



which occurs in the fat of butter.

Now an amino-acid is simply an acid in whose molecule a hydrogen atom of the main radicle has been replaced by an amino-group, $\cdot\text{NH}_2$. We say a hydrogen atom of the main radicle because, of course, the $\cdot\text{COOH}$ group must remain intact as it is the characteristic group in the molecules of these, and of most, organic acids. The amino-acids that are of interest to us at present as being the more important products of the breakdown of protein molecules are amino-derivatives of these fatty acids. Thus from acetic acid we obtain amino-acetic acid,

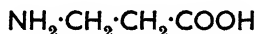


which is usually called **glycine** and sometimes glycoll on account of its sweet taste (Gr. $\gamma\lambda\upsilon\kappa\acute{\upsilon}\varsigma$ = sweet). When we

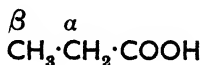
come to consider the formula of propionic acid we at once realise that we can insert the amino-group either in the place of one of the hydrogen atoms of the $\cdot\text{CH}_2\cdot$ group thus :—



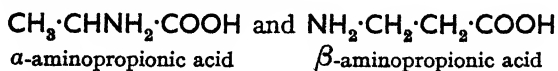
or in place of one of the hydrogen atoms of the terminal $\text{CH}_3\cdot$ group, so :—



We have thus theoretically two distinct aminopropionic acids differing in chemical structure and properties. Both these substances are, in fact, well known. In order to distinguish between such closely related substances and to indicate the position of the amino- or other substituting group in the molecule, a convention is adopted whereby the carbon atoms in a molecule containing a long straight chain are labelled in order with the letters of the Greek alphabet. Furthermore, it is agreed to commence this labelling with the carbon atom next to the characteristic group in the molecule. In the case of an acid this is the $\cdot\text{COOH}$ group, so that, according to this nomenclature, the atoms in the molecule of propionic acid will be designated thus :—



For a longer chain, of course, further Greek letters are employed. We refer, therefore, to the two aminopropionic acids respectively as

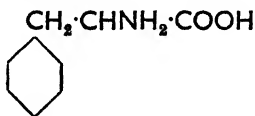


Now while both these substances are of more or less equal interest to the organic chemist, the biochemist has

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relatively little interest in the β -compound, for although it is not completely absent from the body it is not one of the breakdown products obtained from proteins. In contrast with this, α -aminopropionic acid is of such extreme importance from our point of view, that it is given a special name, **alanine**. As a matter of fact it is found that all the amino-acids that are breakdown products of proteins are *alpha*-amino-acids—the amino-group is always attached to the carbon atom next the carboxyl group. There *are* amino-acids that contain more than one amino group—the di-amino acids for instance—and in these we have amino-groups attached to other carbon atoms as well: but, even so, one amino-group always occupies the α -position.

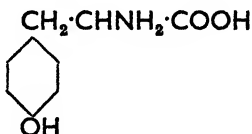
To return to the amino-acid alanine—this is an important substance, not only because it itself occurs very commonly as a constituent of protein molecules, but because also it has several derivatives that are important for the same reason. If we introduce into the molecule of alanine a phenyl group,* C_6H_5 , in the β -position, we obtain **phenylalanine** :—



This, again, is a substance of frequent occurrence as a protein constituent. By the substitution of a hydroxyl


* It should be hardly necessary to remind the student that the six carbon atoms in the phenyl group are arranged in a ring which is usually represented as a hexagon. Each corner of the hexagon then represents a carbon atom, and to each carbon atom not represented as being attached to any other group a hydrogen atom is understood to be linked. Further, there exist not only rings composed of six carbon atoms, but also, though less commonly, those of five or even four. And not only do we know these varieties of

group for the hydrogen atom understood to be attached to the carbon atom in the opposite or "para" position to the side chain, we arrive at the formula for α -amino, β -hydroxy-phenyl-propionic acid :—




the more familiar name of which is **tyrosine**—a word which, being derived from the Greek *τυρός* meaning "cheese," serves to remind us that the particular amino-acid we are considering was first obtained by Liebig as long ago as 1846 by heating up cheese with potash. The making of Welsh rarebit in this fashion has never been


rings composed entirely of carbon atoms, but there are also those which contain one or more atoms of some other element. Nitrogen atoms take their places in many such heterocyclic rings, and whenever they do so it is necessary to write the "N" representing the nitrogen atom across the corner of the ring and not outside it, otherwise it would appear that the nitrogen formed part of a side chain attached to an understood carbon atom, instead of being a constituent atom of the ring itself. Thus two very important heterocyclic rings which we meet among substances of biological importance are the

pyrrole ring , containing one nitrogen atom and four of

carbon, and the **iminazole ring** which is similarly five-membered,

but contains two nitrogen atoms thus:—  Lastly we have

substances whose molecules are composed of two rings having two atoms in common. Of these the most familiar is probably naphthalene—the so-called "camphor," which is extensively used to ward off moths from clothes. Its formula is represented as two benzene

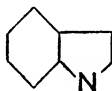
rings with two carbon atoms in common:—  But we

shall also meet substances in which the two fused rings are not, as in this case, similar.

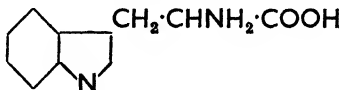
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popularised, but sufficient attention has been given to it to make it evident that in Liebig's experiment there occurred a breaking down of the chief protein of the cheese, namely, **casein**, into its constituent amino-acids, of which tyrosine, on account of its stability and relative insolubility, happened to be the one most readily isolated from the melt.

There are other ring derivatives of alanine which occur among the constituents of the protein molecule. On page 6 (footnote) we reminded the reader that many conjugated ring structures were known. Of these there is one known as the **indole** ring, which is composed of a benzene ring and the heterocyclic pyrrole ring :—

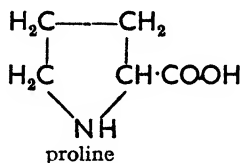


When this is substituted in the β -position in the molecule of alanine, β -indolepropionic acid results :—

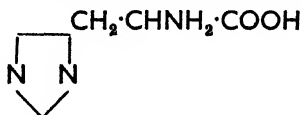


This substance was named **tryptophane**, even before it was isolated, from the circumstance that it is liberated (Gr. *φᾰνός* = bright, evident) in a tryptic (pancreatic) digest of a protein, and can there be detected by the pink colour it gives, *when in the free condition, but not when in combination*, on the addition of bromine water. Tryptophane is decomposed by boiling with dilute acids and so cannot be obtained from proteins by treatment with strong reagents. It is therefore not surprising that it should not have been isolated until in 1901 Hopkins and Cole, working

in Cambridge, submitted the milk protein casein to the gentler disruptive action of pancreatic extract, and by the use of a suitable method of precipitation succeeded in separating the resulting products. While these workers were engaged upon this task, news arrived from Berlin that Emil Fischer was also isolating a new product of protein hydrolysis. Furthermore, Fischer's new substance was found to be a white crystalline solid, just as was the product obtained by Hopkins and Cole. But when Fischer published his results, it was seen that the compound he had obtained was not tryptophane, nor, indeed, a true amino-acid at all, but a substance called **proline**, in which the nitrogen atom is not present in an NH_2 group, but forms part of a "pyrrolidine," i.e. reduced pyrrole, ring :—



We must mention one other important derivative of alanine which contains in the β -position a five-membered ring containing two nitrogen atoms and known as the iminazole ring. This iminazole-alanine, then, has the formula :—



It is the amino-acid more familiarly known as **histidine** which is usually obtained by the hydrolysis of blood proteins.

We have now mentioned sufficient typical amino-acids to give an idea of the range of variation of structure

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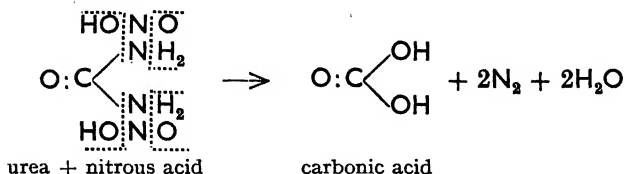
found in the group. We need, however, to consider in more detail the relationship between these amino-acids and the fully-formed protein molecule. The fact is that if we take *any* protein and disrupt its molecule by boiling with a dilute acid we find that we obtain a mixture of amino-acids, all of which are α -amino-acids, and among which are included all those we have already mentioned, together with about a dozen more. Of these remaining ones we shall describe in detail a few further examples later. This decomposition into amino-acids is found to occur with all proteins, whether of flesh or blood, plant or animal; we may say, therefore, that the amino-acids are the units of which protein molecules are composed—that they represent, so to speak, the bricks of which the complicated edifices are built. We have said that the majority of proteins give the same score, or so, of amino-acids on decomposition. But the various proteins yield these same amino-acids often in widely differing proportions, so that we may say that while all protein molecules consist of complexes of amino-acid molecules, the ratio in which these constituents are combined in one protein is very different from that in which these same substances occur in a second. In special cases a particular protein may contain an abundance of a given amino-acid: another protein may contain almost none of it.

A knowledge of this general principle gives the key to the whole of the later study of the behaviour of proteins in the body. It also has important bearings on a topic that we will now discuss, namely, the qualitative tests for proteins. It is evident that it is of the utmost importance to be able to decide whether or no any given biological fluid contains a protein, and some of the most valuable evidence on this question is furnished by certain

colour reactions that proteins give. Of these we will describe that known as **Millon's reaction** first. If a few drops of Millon's reagent be added to a solution containing a protein a white precipitate is formed, and this precipitate turns a brick-red colour on boiling cautiously. It has been found that this colour reaction is given also by tyrosine, and, indeed, by all substances which, like tyrosine, contain the hydroxyphenyl ring. In fact, the reason why practically every protein gives Millon's test is that tyrosine is of almost universal occurrence in proteins. But any protein that contains no tyrosine will not give Millon's reaction. Such a protein is **gelatin**, which in the pure condition does not give the test, although the commercial product is usually contaminated with traces of some impurity which does give a faint reaction. Incidentally, we must call attention to the fact that it is absolutely necessary that a red colour should be produced on boiling, the white precipitate not being given by all proteins. And, again, many substances of physiological importance, including even urea, give with Millon's reagent a white precipitate, which, however, does not show the colour change on heating. In order to understand this we must know that Millon's reagent is a mixture of the nitrates of mercury, obtained by dissolving the metal in strong nitric acid. It also contains excess of the nitric acid and a variable quantity of nitrous acid formed by the reduction of the nitric. Now when urea is added to solutions of mercury salts, white precipitates, composed of basic double salts of urea and mercury; are produced. This accounts for one aspect of the behaviour of urea towards Millon's reagent. But there is a second; for whenever a substance containing the amino-group is brought into contact with nitrous acid, a reaction occurs

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that leads to the liberation of nitrogen. In the particular case of urea this takes place as follows :—



carbonic acid being formed, and further decomposing into carbon dioxide and water. We do not wish to elaborate this matter further than to point out that the mere production of a white precipitate with Millon's reagent is no certain test for the presence of tyrosine or a protein unless a red colour appears on boiling, and that if, instead, the reaction is accompanied by effervescence, then it is probable that urea may be present.

A second colour test given by the proteins is that known as the **glyoxylic reaction**. The reagent is prepared by reducing oxalic acid with sodium amalgam or magnesium powder. There is thus obtained a solution whose

essential constituent is glyoxylic acid, $\begin{array}{c} \text{CHO} \\ | \\ \text{COOH} \end{array}$. A quantity

of this "reduced oxalic acid" or "glyoxylic reagent" is mixed with some of the solution to be tested. Then a layer of strong sulphuric acid is allowed to run down the side to the bottom of the test-tube, and if a protein be present a purple ring forms at the junction of the two layers of liquid. This reaction is associated with the presence of tryptophane; indeed, immediately tryptophane was isolated by Hopkins and Cole it was found to be the constituent of the protein molecule that is responsible

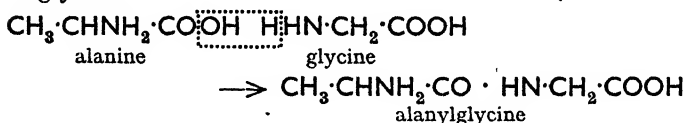
for the formation of the purple colour under these conditions. Naturally, then, any protein from whose molecule tryptophane is absent will fail to give this test, and again we find that this is the case with gelatin. It gives absolutely no colour with glyoxylic acid in the presence of strong sulphuric acid. The student will have realised by this time that these two so-called colour tests for proteins are simply tests for the amino-acids tyrosine and tryptophane respectively, and that these reactions can be used as tests for proteins merely because tyrosine and tryptophane are of such common occurrence in protein molecules.

Further, it is the presence of aromatic ring structures such as are found in tyrosine, tryptophane and phenylalanine that is responsible for the **xanthoproteic** reaction which is given by all proteins. Strong nitric acid forms yellow nitro-derivatives of these ring structures, the colour of which is turned to orange on addition of ammonia. The first of these facts is a matter of everyday experience to anyone who allows drops of strong nitric acid to come into contact with the proteins of his skin. Gelatin responds to the xanthoproteic test owing to the presence of phenylalanine.

We turn now to consider the question as to the manner in which amino-acids are combined to form protein molecules. Since the amino group and the carboxyl group of an amino-acid are the two most reactive groups in the molecule it would be expected that linkage of amino-acid molecules would be brought about by means of these two groups. Evidence that this is the case is furnished by a number of observations. For example, amino-acids, like other substances containing the NH_2 group, evolve nitrogen when treated with nitrous acid. Now

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when a protein is treated with this reagent very little nitrogen is evolved : this indicates that the amino-groups of the constituent amino-acids are not free in the protein molecule, and that they are therefore concerned in forming the linkages that serve to unite these units into the whole complex. And, indeed, Emil Fischer succeeded in causing amino-acid molecules to unite to form long complex chains by the elimination of water between the amino-group of one molecule and the carboxyl group of the next. In practice this result has to be achieved by somewhat roundabout methods, but this circumstance does not affect our present argument. Let us take the case of the condensation of a molecule of alanine with one of glycine :—



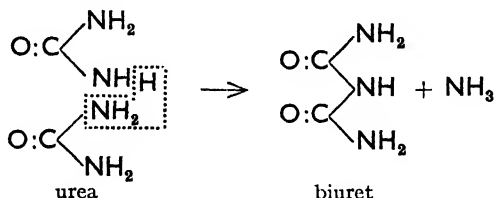
In the resulting product it will be seen that there is still an amino-group and still a carboxyl group both of which are available for forming linkages, in a precisely similar manner, with other amino-acid molecules, so that by a repetition of the appropriate chemical manipulations compounds whose molecules consist of chains of amino-acids can be built up.

From this mode of formation it will be seen that the characteristic group whereby the amino-acid molecules become linked together is $\cdot\text{CO}\cdot\text{NH}\cdot$. This is called the **peptide linkage**, and the substances prepared in the way we have described are referred to as di-, tri-, tetra-, or poly-peptides, according to the number of amino-acids united in their molecules. Many such polypeptides have now been synthesised, and it is found that in general the

more amino-acid molecules that are condensed into a single long peptide chain the more closely is the resulting compound found to resemble in solubility and other chemical properties the simpler naturally-occurring proteins. More important still, it is found that these "artificial" polypeptides — provided that they are built up of amino-acids known to occur as constituents of the natural proteins — can be re-hydrolysed to their constituent amino-acids by the action of those very same digestive ferments that, as we shall see, bring about in the alimentary canal a similar decomposition of the proteins of the food, and from what we shall later learn about the specificity of the action of enzymes it will be realised that this can only mean that the same chemical grouping is being attacked in both cases ; in other words, that the essential grouping in the naturally-occurring proteins as in the artificially-made polypeptides must be the peptide linkage. The longest polypeptide chain manufactured so far consists of nineteen amino-acid units ; this is as far as it has been found possible up to the present to proceed in the direction of the synthesis of protein molecules. But there is no reason to doubt that further progress will be achieved when yet more efficient methods of bringing about this condensation are devised, and also—probably more important still—when we possess information which will enable us to choose from among the enormous numbers of possible arrangements of these amino-acid molecules those which actually occur in the structure of the proteins. The difficulty is that the number of theoretically possible combinations and permutations of even a score of amino-acid units reaches figures of astronomical magnitude, and the task of deciding between these is not made easier by the circumstance that we do

not know any way of breaking down proteins just as far as to di- and tri-peptides in which the order and arrangement of the amino-acids, being easy to discover, would furnish a clue as to which amino-acids were adjacent in the original protein molecule. All our decompositions of proteins seem either to stop at products that are still too complex for us to investigate directly, or to go right down to the individual amino-acids. But we do at least know this that all the various proteins are larger or smaller chemical complexes of amino-acids between which at all events the chief connecting group is the peptide linkage.

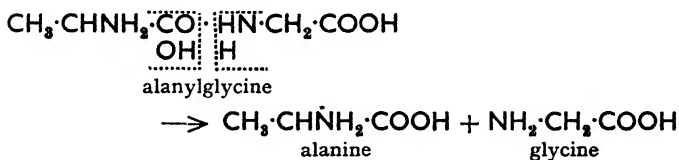
We are now in a position to understand a fourth reaction that is important as a means of detecting the presence of proteins. It consists in the formation of intensely-coloured copper derivatives—usually blue or purplish, but in some cases pink—when a drop of dilute copper sulphate solution is added to a protein solution made strongly alkaline with excess of caustic soda. This is known as the **biuret test** because it was first used as a test for biuret—a substance produced by heating solid urea, two molecules of which react with the evolution of ammonia:—



But this colour reaction is by no means confined to biuret itself; it has been found to be given by practically all compounds that contain two $\cdot\text{CO}\cdot\text{NH}\cdot$ groups united to the same carbon (or nitrogen) atom. This, of course, includes biuret itself, in which the central nitrogen

atom unites the two $\cdot\text{CO}\cdot\text{NH}\cdot$ groups, and all the proteins and polypeptides, where the union is brought about by a carbon atom. Naturally gelatin, in common with all other proteins, contains many such peptide linkages, and in virtue of this it gives the biuret reaction well. We might mention here that gelatin, in spite of its deficiencies in other directions, contains glycine in such abundance as to be used as a source of it in the laboratory.

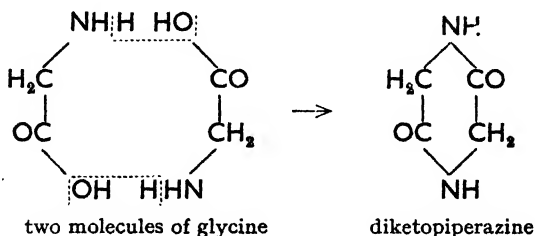
We have explained the evidence on which is based the view that the protein molecule is composed of a complex of amino-acids condensed together by the elimination of water. It is now easy to see that the reaction which occurs when a protein is heated in acid solution is the reverse of condensation: it is a process of hydrolysis in which water molecules are added to the peptide linkages and the free amino-acids are re-formed. Taking our original example of alanylglycine we may write:—



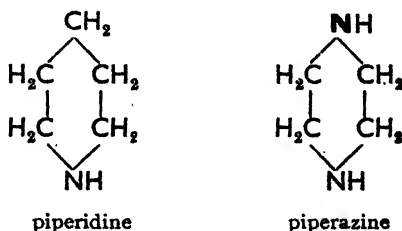
Of recent years, however, a number of workers have put forward the idea that the simple chain-like peptide linkage is not the only one that unites amino-acids in the protein molecule, and that these units may also be associated in complex cyclic structures. One way in which this could take place is very easy to understand. We have already said that when two amino-acids form a peptide linkage between the amino group of one and the

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carboxyl group of the other the remaining amino and carboxyl groups will be left free. Suppose now that these two remaining groups also unite in a peptide linkage ; we then obtain a heterocyclic compound with a ring composed of four carbon and two nitrogen atoms. Let us illustrate this by taking glycine as the simplest example :—



The resulting ring compound is called **diketo-piperazine** because it can be regarded as derived by the introduction of two keto, or carbonyl, groups, :CO, into the molecule of the parent substance piperazine. And piperazine itself may be regarded as a derivative—in which an extra “azo-,” i.e. nitrogen atom, has replaced a carbon link in the ring—of piperidine, a substance that derives its name from the circumstance that it can be obtained from pepper (Lat. *piper*).

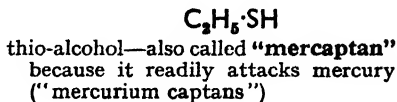
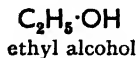


Piperidine itself is obtainable by reduction of that most fundamental of all heterocyclic compounds—pyridine.

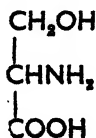


It will be evident that not only glycine, but any two α -amino-acids can theoretically couple up in this double fashion and produce substituted diketopiperazines. In fact, an α -amino-acid may be regarded as one-half of a diketopiperazine molecule. Such compounds have actually been obtained by the hydrolysis of proteins, but the extent to which they exist pre-formed in the protein molecule is still a matter for debate.

We have yet to mention that practically all proteins contain sulphur. Much of this sulphur is present in the form of a sulphur-containing amino-acid called, on account of its discovery in urinary bladder concretions, **cystine** (Gr. *κύστις*. = bladder). We shall understand the formula for this substance most easily if we remember that a divalent sulphur atom can often replace an oxygen atom in a compound and so give rise to a thio-derivative. Among organic compounds we have such as :—

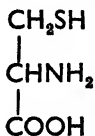


Now in a similar way the β -hydroxy derivative of alanine, namely

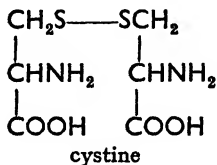


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has a corresponding thio-derivative with the formula



This is called *cystein*. On exposing a solution of this substance to the air the hydrogen atoms of the sulphydryl groups of each of two molecules are oxidised to water, and by the union of the two resulting residues we obtain the complex sulphur-containing amino-acid cystine itself :—



Cystine is abundant in egg albumin and in **keratin**, the insoluble protein of hair, wool and horn ; it is by the hydrolysis of these latter materials that it is usually obtained.

The presence of “cystine” sulphur in a protein can be readily demonstrated by boiling it with strong caustic soda solution. Sodium sulphide is then produced and can be detected by the addition of a solution of a lead salt, when insoluble black lead sulphide is precipitated.

Another sulphur-containing amino-acid that has more recently been found to be abundant in proteins is **methionine**: it is α -amino, γ -methylthiol-butyrac acid:

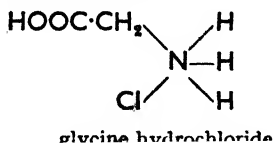
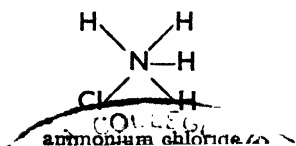


As much as 90 per cent. of the sulphur in some proteins (for example ~~the casein of milk~~) is in this form. Its

presence, however, cannot be shown by the sulphur reaction as it is not decomposed by alkalis.

The student by this time will have realised that this and the other colour reactions we have described are by no means specific for proteins as such, but are simply tests for various fragments of the protein molecule. All of them *except the biuret reaction* would be given by a mere *mixture* of the appropriate amino-acids, but, as will be evident from the next chapter, such a mixture would differ very considerably in its solubility relationships from a protein itself.

Now that we have become acquainted with all the amino-acids we need to know for our present purposes we must refer to some of their more general properties. In the first place we must ask the student to visualise all these amino-acids as white crystalline substances differing very much in the "definiteness" of their behaviour from the complex proteins from which they are derived. By virtue of the fact that they contain the carboxyl group they behave as acids and form crystalline salts with bases. For example, we can readily obtain the sodium salt of glycine, $\text{NH}_2\cdot\text{CH}_2\cdot\text{COONa}$. But these amino-acids contain also the amino-group which is basic in character and confers upon the substances containing it the property of acting as bases and forming salts with acids. Thus glycine reacts with hydrochloric acid to form a crystalline hydrochloride of the formula $\text{HCl}\cdot\text{H}_2\text{N}\cdot\text{CH}_2\cdot\text{COOH}$, which may be regarded as a derivative of ammonium chloride, thus :



A substance which possesses this property of acting as a base towards acids and as an acid towards bases is said to be **amphoteric**. On account of this simultaneous presence of acidic and basic groups, amino-acids are not strong enough to be estimated directly by titration with standard acid or alkali ; they do not give a sudden change of reaction at the end-point. But if they are first treated with formaldehyde this substance reacts with, and so masks, the amino-group (forming a methylene derivative) and the amino-acid can then be estimated by titration of its carboxyl group with alkali in the ordinary way. Whole protein molecules are also amphoteric, because in them not quite all the amino- and carboxyl groups are used to form peptide linkages so that a few remain free—a circumstance that accounts for much of the characteristic physico-chemical behaviour of proteins.

In conclusion, we will point out that it is the problem of modern protein chemistry to determine the exact number of molecules of each of the amino-acids that go to make up the various proteins and the order in which these are combined. But as a matter of fact for no protein have we at the present time such complete information. The separation and estimation of all the amino-acids present in the hydrolysis mixture of a protein is such a tedious process that quite commonly one has to be content, for the purposes of identifying a protein, with a knowledge of the relative amounts of each *class* of amino-acid it contains. This "nitrogen distribution" in a protein is determined by Van Slyke's method, which is roughly as follows. The protein is hydrolysed by boiling with 20 per cent. hydrochloric acid ; its amino-acids will thereby be liberated, but the tryptophane will be decomposed to a brown insoluble "humins." The total nitrogen in this is

estimated by Kjeldahl's method (incineration with strong sulphuric acid and distillation into standard acid of the ammonia formed) and is taken to represent the tryptophane nitrogen of the protein. The di-amino-acids of the hydrolysis mixture are precipitated from the mono-amino-acids by phosphotungstic acid. In each of these fractions the total nitrogen is determined (Kjeldahl) and also the volume of nitrogen evolved on treatment with nitrous acid (pp. 11, 13). This latter portion of nitrogen represents the amino-groups and the difference between it and the total nitrogen of the same fraction represents the nitrogen that forms part of heterocyclic structures such as proline. Lastly, cystine (which is precipitated with the di-amino acids) can be estimated by complete oxidation and determination of the sulphate formed. By the method thus briefly outlined it is possible to account for all but one or two per cent. of the total nitrogen contained in the original protein. But even so, this does not tell us the order in which the amino-acids are arranged, nor does it enable us to detect by purely chemical means the small but significant differences in structure between the similar proteins of different species. The immunological reactions by which an organism distinguishes between, say, the egg albumin of a hen and that of a duck are unrivalled in sensitiveness and specificity by any chemical technique.

SUMMARY

We may now summarise the contents of this chapter in a schematic form thus :—

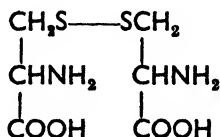
Proteins are the characteristic constituents of living matter. Their molecules are built up of large numbers of amino-acids united by peptide linkages into straight chains and possibly cyclic complexes. Of these amino-acids, important examples are :—

amino-acetic acid, glycine, $\text{NH}_2\cdot\text{CH}_2\cdot\text{COOH}$,

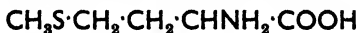
α -amino-propionic acid, alanine, $\text{CH}_3\cdot\text{CHNH}_2\cdot\text{COOH}$,

with its derivatives phenylalanine, tyrosine, tryptophane and histidine, whose relationships are shown on the next page.

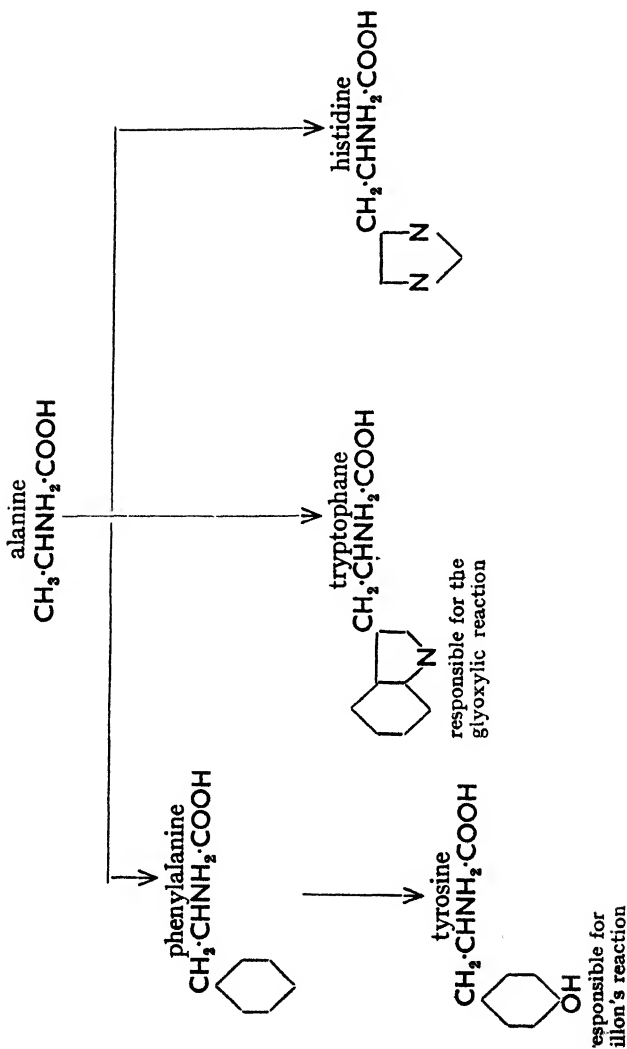
The sulphur contained in the protein molecule is partly in the form of the complex thio-amino-acid cystine :



and partly in the form of methionine:



The biuret reaction given by proteins is due to the presence of the linkage $\cdot\text{CO}\cdot\text{NH}\cdot\overset{\cdot}{\underset{\cdot}{\text{C}}}\cdot\text{CO}\cdot\text{NH}\cdot$



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The range of theoretically possible variations of structure in proteins is treated of mathematically by LEATHES in an article entitled:

"Function and Design" (British Association Reports, 1926; reprinted in *Science*, vol. 64 (1926), p. 387).

The original account of the isolation of tryptophane by HOPKINS and COLE is contained in a paper entitled:

"A Contribution to the Chemistry of the Proteins." Part I. "A Preliminary Study of a hitherto undescribed Product of Tryptic Digestion" (*Journal of Physiology*, vol. 27 (1901), p. 419).

This article should be read by all students as an example of the way in which such a problem is dealt with in research.

Full details of the methods of carrying out the tests for proteins are given in

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CHAPTER II

THE CHIEF VARIETIES OF PROTEINS: THEIR PROPERTIES AND RELATIONSHIPS

"This will teach you their names, the ingredients they're made of,
And which to indulge in, and which be afraid of."

—*Ingoldsby Legends*.

WE have learnt in a general kind of way that a protein molecule is a complicated structure built up of amino-acid molecules. It is now necessary to point out that these chemical aggregates of amino-acids may vary very considerably in their complexity, so that there comes to be a large variety of protein molecules ranging from those that contain comparatively few amino-acid molecules, and are relatively small and simple, to others whose molecules are enormous compounds of large numbers of these chemical units. With these varying degrees of complexity are associated well-marked differences of chemical and physical properties, which make it possible to classify this wide range of substances into subsidiary groups, and so to deal with them systematically and scientifically. The most important property which is utilised for the separation, and, indeed, in many cases for the definition, of the chief classes of the proteins, is that of solubility. Our task in this chapter thus resolves itself into a study of the various kinds of proteins and of their solubilities, and of the method whereby this property is used in their identification. If Chapter I may be regarded from some points of view as supplying the answer to the questions "What is a protein?" and "How does one determine

whether there is a protein in a given mixture?" the present chapter may be said to carry the investigation further, and to indicate how to meet a demand for information as to the nature of a protein already known to be present.

When we attempt to sort out the protein constituents of cells and tissues and to study the kinds of proteins that occur "native" in living matter we at once meet two main groups of proteins similar in many of their general characteristics but differing in their solubilities. The proteins of the one group are called **albumins** after egg albumin, a typical member of the group; those of the other group are named **globulins** because the chief constituents of blood "globules," i.e. corpuscles, were at one time supposed to be proteins of this kind.

These two groups of proteins are separated and defined by means of the marked differences in their solubility relationships. On the whole the globulins show less tendency to go into or to remain in solution than do the albumins. This is at once seen in their behaviour towards strong solutions of neutral salts. In concentrated solution, dissolved neutral salts seem to have a kind of attraction for the solvent water, so that they have the power, so to speak, of robbing the solvent from another dissolved substance such as a protein, and so of precipitating it. Doubtless this process of "salting out" involves much more complicated relationships than this simple statement would indicate; but, neglecting other factors, the precipitating power of a salt is determined to a great extent by its concentration, so that the most useful precipitants are those that can be obtained in greatest concentration, that is which are most soluble. For this reason ammonium sulphate is most conveniently used for the purpose. It is

found that if to a solution of a globulin there is added such an amount of ammonium sulphate as is just half of the quantity which would be required to saturate the solution, the globulin is precipitated by the withdrawal of the water. This "half-saturation with ammonium sulphate" is most easily performed by adding to a given volume of the protein solution an equal volume of a saturated solution of the salt. The globulin so precipitated has not undergone a chemical change, and will readily pass into solution again as soon as the excess of ammonium sulphate has been washed away from it. On the other hand, the albumins require more than half the saturation concentration of ammonium sulphate to throw them out of solution, so it is customary to precipitate them by completely saturating the solution with the salt. This, of course, can be done only by shaking with the solid crystals of ammonium sulphate; it is obvious that the addition of no matter how much saturated ammonium sulphate solution to a liquid not already saturated with the salt can never result in a mixture in which the salt is present in saturation concentration.

The difference in solubility relationships between albumins and globulins is shown further by the fact that while the albumins dissolve easily in distilled water the typical globulins will not dissolve unless a small amount—1 per cent. or so—of sodium chloride or other neutral salt be present. So that when serum or other salt solution containing globulins is dialysed these substances are gradually precipitated as the salt is removed. As a matter of detail we ought to mention that, although the behaviour we have described is shown by the typical globulins, there are found in animal tissues many exceptional or **pseudo-globulins** which resemble the albumins in being soluble

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in distilled water. A part of the globulin fraction of blood serum is, for example, soluble in distilled water. But all globulins differ from albumins in being precipitated by half-saturation of their solutions with ammonium sulphate. In solutions that are faintly acid (1 per cent. acetic) or faintly alkaline (2 per cent. sodium carbonate) globulins as well as albumins dissolve easily even in the absence of neutral salts.

A very characteristic property common to both albumins and globulins, but shared by no other proteins, is that of coagulating when their solutions are heated to a certain temperature. This process of **coagulation** results in a removal of the protein from solution in a manner that differs from the precipitation by ammonium sulphate already mentioned, inasmuch as on coagulation the protein molecule undergoes a partial decomposition, which we shall describe later. Coagulation by boiling furnishes us with a method of separating albumins and globulins from all other proteins.

By partially decomposing the native proteins in various ways we obtain a number of simpler proteins of smaller molecular weight known collectively as "derived" proteins. If we bring about such a decomposition by the use of very dilute acids (0.4 per cent. hydrochloric at body temperature) or alkalis we obtain what are called **meta-proteins**, i.e. changed proteins. These metaproteins differ from the native proteins from which they are derived by being soluble only in markedly acid or alkaline solutions. They are precipitated on neutralisation, for neither pure water nor neutral salt solutions will dissolve them. In other words, there is a certain narrow range of reaction just on the very faintly acid side of the neutral point in which these metaproteins are insoluble. The

metaproteins are also insoluble in solutions half-saturated with ammonium sulphate. The great importance of the metaproteins as a class lies in the fact that they are formed by partial hydrolysis when albumins and globulins are heated with water, and indeed form the coagulum which these native proteins yield on heating. This explains why the process of coagulation is an irreversible one, for it has involved a conversion of the original albumins or globulins into metaproteins. Furthermore, we can now see that in order to remove a coagulable protein completely and cleanly from solution, the coagulation must take place in very faintly acid solution. For in such a solution the metaprotein formed is insoluble. But if the solution be more acid or more alkaline than corresponds to the condition of minimum solubility of the metaprotein, this, though produced, will remain in solution, and no coagulum will be formed. The application of this fact to the analysis of protein solutions is evident. For example, it is useless to try this formation of a coagulum on boiling as a test for albumins in urine, without first bringing the fluid to the appropriate faintly acid reaction, particularly if it has become alkaline on standing. And, in general, if the reaction of the liquid under examination is not appropriate, then the metaprotein will not be completely carried down in the coagulum, and will give trouble at later stages of the analysis. Fortunately, in chlorphenol red and bromcresol purple we possess indicators whose turning-points mark the correct reaction for the purpose.

Another way of bringing about a partial decomposition of native proteins is by submitting them to digestion. If we add an extract of stomach containing the protein-digesting enzyme pepsin to a solution of a native protein

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and keep the mixture at body temperature for some hours we obtain a number of derived proteins which we can separate by their differing solubility relationships as follows. Firstly, we neutralise the mixture to precipitate metaproteins and then boil the filtrate in order to coagulate and remove any unchanged albumins or globulins. We then half-saturate the solution with ammonium sulphate, and obtain a precipitated fraction known as **primary proteose**. Having filtered this off, we then fully saturate the filtrate with ammonium sulphate, and obtain a fraction which consists of **secondary proteose**. According to whether an albumin or a globulin was the native protein taken for digestion the particular proteoses formed would be termed albumoses or globuloses, and so on in the case of the digestion products of other proteins. Even after complete saturation with ammonium sulphate some proteins remain in solution. These are the **peptones**. They are the only proteins that can withstand the precipitating action of so strong a neutral salt solution as this, so that all other proteins may be separated from these by full saturation with ammonium sulphate. In other words, if a solution be saturated with ammonium sulphate and filtered, and if by means of the colour reactions a protein is found to be present in this filtrate, that protein must necessarily be a peptone.

Incidentally, we must remind the student that several of the protein colour reactions described in Chapter I are due to the presence of particular amino-acids in the protein molecule, and that the same colour reactions are given by these amino-acids in the free condition. But the free amino-acids are much more readily soluble substances than the proteins; they are not precipitated by ammonium sulphate and other reagents which bring

down the proteins, nor do amino-acids give the biuret reaction, for they contain no $\cdot\text{CO}\cdot\text{NH}\cdot$ group. This last fact serves to distinguish free amino-acids from peptones, which give a pink colour in the biuret reaction.

So far we have been passing from the complex to the simpler proteins. We shall now pass in the opposite direction to substances more complex than any that we have yet mentioned. There are many substances whose molecules consist of a compound of a complete protein molecule with some other substance that is not a protein. These substances are called **conjugated proteins**. A very familiar example is hæmoglobin—the red respiratory pigment of the blood. This consists of a coagulable protein called globin, united to a non-protein molecule called hæm. In some conjugated proteins the non-protein part of the whole complex is phosphoric acid. In these cases we are dealing with the **phosphoproteins** of which **casein**, the chief protein of milk, is an example. Or, again, the non-protein group may be a carbohydrate such as a sugar. In this case the substance belongs to the **glucoproteins**. **Mucin**, the substance which gives the slimy lubricating properties to many mucous secretions, is an example of these. Lastly, we reach the height of complexity in those conjugated proteins that form important constituents of the nuclei of cells and so are termed **nucleoproteins**. In these the non-protein part of the molecule is itself a complex of molecules of phosphoric acid, sugars, and basic substances related to uric acid termed purine bases. We do not wish to describe these nucleoproteins in detail until we treat of their relation to uric acid in Chapter VII. Our main object at the moment is to point out that the solubility relationships of these three

groups of conjugated proteins are all very similar. They are all soluble in dilute alkaline solutions, but in no other of the media we have previously mentioned. They are specially insoluble in dilute acids, so that a liquid whose reaction is acid cannot contain these proteins in solution ; they are therefore precipitated on the change of reaction from alkaline to acid. Mucin is distinguished, however, from nucleoproteins and casein by the fact that it is insoluble in strong acetic acid—a fact often utilised by the histologist for rendering more easily visible the mucin in cells.

The fact that nucleoproteins and casein are soluble in fairly strongly acid solutions (such as 33 per cent. acetic acid) is a point of some considerable significance. Taking casein as our example, we have seen that this protein is soluble in dilute alkalis, insoluble in neutral and faintly acid solutions, but again soluble in more strongly acid solutions. In other words, just as in the case of the meta-proteins, there is a certain range of reaction over which the solubility of casein is very small and within this range one particular reaction at which solubility is a minimum. This is related to the amphoteric behaviour of proteins already referred to in our first chapter (p. 22). In alkaline solution the protein may be regarded as acting as an acid and as forming with the alkali a salt that is much more soluble than the free casein itself. In the strongly acid solutions the protein acts as a base, and here also forms an easily soluble salt with the acid. But at one particular intermediate reaction the casein acts neither as an acid nor as a base ; it remains as free, practically insoluble, casein. We have seen that the albumins and globulins, on the other hand, are soluble in solutions ranging from acid to alkaline in reaction, but even here we have pointed out

that their typical property of coagulating on heating shows itself completely only at that particular reaction at which the coagulated protein has its minimum solubility.

The incomplete protein gelatin furnishes another interesting example of the influence of the reaction or, as we should strictly say, the hydrogen ion concentration, of the medium on the solubility of proteins. As is well known, gelatin is soluble enough in water, dilute salt solutions, acids and alkalis, if these are warmed, but a solution containing more than about 1 per cent. of the protein gelatinises on cooling. There is here at first sight no one concentration of hydrogen ions at which the solubility is a minimum. But gelatin in common with all other proteins (apart from a few unusual ones found in cereal grains) is insoluble in moderately strong alcohol, and it is found that if an equal volume of alcohol is added to each of a series of solutions of gelatin with reactions varying progressively from markedly alkaline to markedly acid the greatest precipitation of gelatin occurs in one particular solution—in that in which the hydrogen ion concentration corresponds to the condition of minimum solubility—while less gelatin or none is precipitated from the solutions more acid or alkaline than this. In a later chapter (p. 406) we shall have more to say about this extremely important influence of hydrogen ions on protein solubility but the present account will serve as an introduction to the subject. For completeness we should just mention that gelatin is precipitated by half-saturation with ammonium sulphate.

This brings us to the end of our survey of the chief groups of substances included under the term proteins and of their solubility relationships. Let us once more

emphasise the circumstance that these solubilities serve not merely for the analytical separation, but also for the definition of the various groups, for we do not as yet know enough about the structure of protein molecules to be able to classify them more systematically according to their more definitely chemical relationships. There is no question at present of our giving a chemical formula to even the simplest protein.

On account of the extreme importance of the separation of the various types of proteins in biochemical work we summarise on page 38 the solubilities we have already mentioned—in the form of a diagram in which a line drawn on a level with the name of a particular protein and below that of one of the solvents indicates that that protein is soluble in the particular solvent, whereas, where the space is left blank the protein is insoluble in, or in the case of the ammonium sulphate solutions, is precipitated by, the particular medium. The student will realise that this scheme will tell him what proteins may be present in (say) an acid solution or in a solid mixture that is completely soluble in dilute acid, and will give him all the information he requires in the separation of the groups of proteins we have mentioned. The exact way in which this information is to be used for this purpose can probably be devised by the reader himself. In any case, it is fully described in works devoted to practical physiological chemistry. Besides giving our summary in this semi-diagrammatic form we shall mention that the only methodical way in which a qualitative analysis of any kind can be carried out consists in noting down at each stage every substance which may possibly be present in each filtrate or precipitate; then, as the analysis proceeds, and more and more of these possible constituents are

proved to be absent they are struck off from the list until only those remain which are actually present. We need hardly remind the student that he can at any stage determine whether he has already exhausted all the proteins of his solution by trying the protein colour reactions.

SUMMARY

THE SOLUBILITIES OF THE CHIEF CLASSES OF PROTEINS

		Distilled water	Dilute salt solutions (1% NaCl)	Dilute acids (1% acetic)	Dilute alkalis (2% Na_2CO_3)	Half- saturated ammonium sulphate	Fully- saturated ammonium sulphate
Typical native proteins, coagulated on heating	Albumins	<hr/>					
	Globulins	<hr/>					
Products of peptic digestion	Metaproteins	<hr/>					
	Proteoses (1)	<hr/>					
	(2)	<hr/>					
	Peptones	<hr/>					
Conjugated proteins	Mucin	<hr/>					
	Casein	<hr/>					
	Nucleoproteins	<hr/>					
Gelatin		<hr/>					
(on warming)							

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A very readable account of the properties of the individual groups of proteins is to be found in

PLIMMER, R. H. A.: *Practical Organic and Biochemistry*. (London: Longmans, Green & Co.)

The appropriate chapters of the books by JORDAN LLOYD and GORTNER (referred to on p. 26) are also useful in this connection.

For recent work on the proteins of blood plasma, see

RIMINGTON, C.: "*Recent Advances in the Chemistry of certain Blood Constituents*" (*Ergebn. d. Physiol.*, vol. 35 (1933), p. 712).

A scheme for the separation of the various proteins is given in the *Practical Physiological Chemistry* by COLE, already referred to at the end of Chapter I.

CHAPTER III

THE DIGESTION OF PROTEINS

“Damit ein Heiligthum aufgerichtet werden kann, muss ein Heiligthum zerbrochen werden.”—*Nietzsche*.

So far we have been considering the chemical constitution and properties of the proteins; we have studied their behaviour in test-tubes. But our ultimate object is to investigate the changes that these substances undergo in living tissues; to study the uses to which they are put in the body. Now before any foodstuff is utilised by the body, whether it be a fat, a carbohydrate, or a protein, it is, in general, changed and decomposed during the chemical processes of digestion. The materials with which the tissues have to work are therefore not the unaltered constituents of the diet, but the products arising from these when they are submitted to the action of the powerful digestive juices. In order, then, to understand the behaviour of proteins in the tissues we must first enquire into the nature of the changes which these substances undergo during digestion, and of the simpler compounds that are thereby produced.

This will be to some extent a familiar story. For example, it is a matter of common knowledge that the enzyme that commences the attack on the proteins of the food is the **pepsin** of the gastric juice. In the acid medium furnished by the hydrochloric acid, also secreted by the stomach, this enzyme breaks down the complex proteins of the food—such as the albumins and globulins—into metaproteins and the simpler non-

coagulable proteins called proteoses and peptones—and there its action ends. No matter how long the food may remain in the stomach its proteins will never be decomposed further than into peptones, for this is as far as pepsin can decompose the protein molecule ; in other words, on the peptones it liberates pepsin is not capable of exerting any further action. Leaving the stomach the semi-digested “chyme” passes into the duodenum, where it is made alkaline by the bile, and is acted on by the enzymes of the pancreatic juice. It has long been known that by rubbing up the pancreas with dilute alcohol an extract is obtained that has a very powerful digestive action on native proteins. To the active enzyme contained in this extract the name **trypsin** (from Gr. *τρίψαι* = to rub or grind, i.e. to disintegrate) is given. But if in an animal experiment pancreatic juice is collected direct from the pancreatic duct, it is found to be devoid of action on native proteins. It, therefore, cannot contain trypsin as such, but it does contain a precursor, **trypsinogen**, from which trypsin itself can be readily obtained. This activation of the enzyme evidently occurs during the somewhat vigorous method used for its extraction from the pancreatic tissue; normally it is brought about by a constituent, **enterokinase**, of the intestinal juice secreted by the glands of the mucous membrane of the small intestine, for a mixture of pancreatic and intestinal juices has a powerful digestive action on native proteins. At one time it was thought that this activating action of enterokinase was itself an enzyme action, and consisted in the “liberation” of trypsin from a more complex substance, but it is now known that the amount of trypsin formed under any given conditions is proportional to and limited by the

amount of enterokinase present—and that no matter how long the action of the enterokinase is allowed to continue, one cannot compensate for deficiency in quantity of enterokinase by prolonging its time of action. It would therefore seem that the action of the enterokinase is not in any sense catalytic, but consists in a direct combination with the trypsinogen to form the more complex trypsin. This secretion of the enzyme in an incomplete form, devoid of action on native proteins until it meets the kinase of the intestinal juice, appears to constitute an arrangement whereby the pancreatic tissue is protected against the digestive action of the enzyme it produces. It is important, however, to note that although it is thus without action on the native albumins and globulins the unactivated trypsinogen alone is capable of hydrolysing simpler proteins such as peptones and also many synthetic polypeptides.

In the intestinal juice there occurs yet another proteolytic enzyme **erepsin** (Gr. *ἐρείπω* = I shatter), which is very active in disrupting the peptide linkages of the simpler peptides, but has no action on the higher polypeptides, the peptones, or the native proteins. As a matter of fact, in actual experience it is found that an extract of pancreas will resolve peptones and some other proteins, for example casein, practically completely into their free amino-acids, but this is because, as modern methods of separation have proved, the pancreatic juice contains some erepsin in addition to its trypsin(ogen). In a similar way it is found that the intestinal juice contains in addition to its erepsin a tryptic enzyme, so that its proteolytic action is not confined to the simpler di- and tri-peptides: it will hydrolyse such proteins as peptones and casein as well. The occurrence of these

proteolytic enzymes is thus more complicated than has previously been thought; they do not occur singly in the individual digestive juices, but associated in various proportions. It is not surprising, therefore, that until this was realised an action was often ascribed to one enzyme that in reality was brought about by an admixed one. Nevertheless, the recent work has made it more than ever true to say that in the alimentary canal we possess a series of proteolytic enzymes—pepsin, trypsin, erepsin—which act respectively and specifically on proteins or protein decomposition products of diminishing grades of complexity until finally all the proteins of the food are resolved into their constituent amino-acids.

Now this conclusion is obviously of first-rate importance in the study of the chemical processes occurring in the body, for it emphasises the central position occupied by the amino-acids in that long series of substances that constitute the intermediate steps between the proteins of the food on the one hand and the excreted end-products to which they give rise on the other. Of this we shall soon have much more to say, but meanwhile we wish to point out that the study of these actions of the protein-splitting digestive enzymes is important also for the light it has shed on the structure of the proteins themselves. For example, by suitable processes of titration it has been shown that during the action of each of the proteolytic enzymes, pepsin, trypsin, trypsinogen and erepsin, carboxyl groups and amino-groups are liberated in exactly equivalent amounts, which suggests that each of these enzymes disrupts the peptide linkage and that alone. There is no evidence here that in the protein structure the amino-acids are united by any other linkage, nor is there any indication that either of these enzymes

brings about a mere depolymerisation of amino-acid complexes. Further, it is found that neither of these enzymes has any action on diketopiperazines, from which it would appear that these substances do not enter to any great extent into the make-up of the majority of proteins. On the other hand, neither will either of these enzymes attack the collagen of bones or the keratin of horn, nails and hair, so that the possibility remains that these diketopiperazines do really enter into the composition of these "scleroproteins," and it is significant that it is these very proteins that yield the greatest amounts of diketopiperazines on acid hydrolysis.

Some extremely interesting and important studies have been made of the action of these proteolytic enzymes on synthetic polypeptides of known constitution, and in general it has been found that the accessibility of these substances to the attack of a given enzyme depends partly on the length of the polypeptide chain and partly on the specific nature of the particular amino-acids of which it is formed. But fascinating as they are we cannot make more than a passing reference to these results, for we have yet to consider the relation of this process of protein digestion to the general chemical economy of the whole organism.

So far we have come to the conclusion that proteins can be hydrolysed to their constituent amino-acids as completely by the successive action of enzymes secreted in various parts of the alimentary canal as by mineral acids—and at a much lower temperature. We have now to show that, between them, the proteolytic enzymes of the alimentary canal do actually bring about a similarly complete disruption of the food proteins taken into the body.

This is a convenient point at which to mention that there is a very good reason against believing that in any case peptones are absorbed in any quantity from the alimentary canal. The reason is this—that the peptones, if they get into the blood stream, are poisonous ; they produce a very harmful fall of blood pressure, they destroy the normal coagulability of the blood, and they upset the permeability of the walls of the blood capillaries, so that increased lymph is produced, and symptoms resembling those of nettle-rash result. Furthermore, the peptone is treated as an unwanted substance in the body ; it is got rid of not by synthesis into tissue proteins, but by being passed into the urine, giving rise to the condition of peptonuria. As these effects do not follow the taking of a heavy protein meal we conclude that peptones do *not* represent the form in which the digested proteins are carried in the blood stream.

When biochemists first put forward the idea that the proteins were completely digested to amino-acids, it was at once objected that if the complex proteins are broken down to this extent, then a large portion of the chemical energy of the protein of a meal must be lost during the process ; but actually this is found not to be the case. Hydrolysis is a chemical change which involves very little change of total energy in the reacting substances, and it is a fact that if we take a given weight of a protein and burn it up in the bomb calorimeter, then take the same weight of the same protein, hydrolyse it into its constituent amino-acids, and then burn these in the calorimeter, we obtain almost as much heat from the combustion of the amino-acids as we do from the original protein. So that this process of protein digestion is not wasteful of the chemical energy of the food.

Lastly, we can see a very definite biological significance in this complete breakdown of the food proteins. Not only is it necessary that these should be converted into soluble and diffusible substances that can be transported to the tissues, but these products of digestion must be such as can be utilised by the tissues, that is, they must be **assimilable**. And it is not very surprising after all that in order to convert the complex proteins of the food—the sheep's proteins which we eat as mutton, for example—into the equally complex but widely different proteins that make up the tissues of a man, it should be necessary to break down the food proteins into their constituent units, and from these to build up the elaborate structures of those protein molecules that form the material basis of our own life processes.

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Recent work on proteolytic enzymes:

BERGMANN, M., ZERVAS, L., FRUTON, J. S., SCHNEIDER, F., and SCHLEICH, H.: *"On Proteolytic Enzymes"* (*Journ. Biol. Chem.*, vol. 109 (1935), p. 325).

Lastly, attempts to elucidate the chemical structure of proteins by detailed studies of the action of digestive enzymes upon them are described in the works of GORTNER, JORDAN LLOYD, MITCHELL and HAMILTON, and WALDSCHMIDT-LEITZ, referred to on p. 26.

CHAPTER IV

THE METABOLISM OF PROTEINS : THE USES OF AMINO-ACIDS IN THE BODY

“Erscheinungen, die sich auf chemische Veränderungen, sowohl der Bestandteile der Zelle selbst, als des umgebenden Cytoplastens beziehen; diese kann man metabolische Erscheinungen nennen.”—*Schwann*.

WE are now in a position to consider the **metabolism** of proteins. The breaking down of the proteins of the food into their constituent amino-acids during digestion is a process which, strictly speaking, is not to be included among metabolic changes, for it occurs in the cavity of the alimentary canal—a space morphologically external to the actual tissues of the body. The term metabolism is reserved for such chemical changes as take place in the living cells themselves. During digestion the proteins, and indeed the foodstuffs in general, are merely prepared for the metabolic changes which they must undergo. It is only when the amino-acids have been absorbed from the digestive tract that their metabolism begins. Looking ahead, for a moment, we shall see that the end-products of metabolism appear in the urine, for it is in the urine that most of the waste products produced during the chemical changes in the body are got rid of—with the exception, of course, of carbon dioxide and water vapour, which pass off from the lungs, and also

of such substances as iron and calcium, which are excreted by the large intestine. So that our problem is to trace the whole range of chemical changes intervening between the absorption of the amino-acids and the formation of the constituents of the urine. Not that we can hope to deal with such a subject completely, for some of the stages in the chemical change lead to the building up of living protoplasm, about whose constitution we really know very little; but, nevertheless, there are certain main lines in these processes of intermediary metabolism that we can trace. Incidentally, one ought to mention that it is because the urine contains all the end-products of metabolism that the analysis of this body fluid occupies such an important position not only in biochemistry, but also in medicine; for not only is it in the urine that the products of normal metabolism are accumulated, but here also are to be sought the abnormal products of disordered metabolism that are indicative of disease. If the reader is a medical student this hint should be sufficient to lead him to pay special attention to the nature, detection and estimation of the pathological constituents of urine.

To realise what is involved in this study of the metabolism of proteins we must think for a moment of the uses to which food is put in the body. Under ordinary circumstances the bulk of the food we eat is used as fuel. It comes to be burnt up in order to provide the body with the energy of muscular movement and with the heat required to keep it warm. But there is a second smaller portion of the food which is used for repairing the "wear and tear" of the tissues of the body. Leaving aside, for the time, the question of the material required for growth, and considering only

the fully-grown organism, we find that fragments of its tissues are being continuously broken down and lost. For protoplasm is a very unstable complex, the continued existence of which depends upon the fact that normally it is being built up at one place, so to speak, as fast as it is breaking down at another. The body is thus like any other machine—not only must it be supplied with fuel if it is to run continuously, but also it must be supplied with small quantities of material in the form of spare parts for the replacement of such components of its mechanism as become damaged or worn out during use. These two uses are subserved by the material taken into the body as food. Under ordinary circumstances most of this is used for fuel, but a certain proportion is required for the very important function of repairing the “wear and tear” of the tissues.

Now it is easy to see that in these processes proteins play a unique part. For, since the tissues of the body consist so largely of proteins, it is obvious that it is only the protein part of the food that can supply that material which is required for the replacement of the “wear and tear”—whereas all classes of foodstuffs, including fats and carbohydrates, as well as proteins, can be used as fuel. Fats and carbohydrates cannot build up the tissues simply because these foodstuffs contain no nitrogen.

And not only are the proteins unique in this respect, but they have also another important use in the body, inasmuch as they furnish ready-made the ring groupings which the body is unable to synthesise, but which are indispensable for the manufacture of the nitrogenous constituents of essential internal secretions, enzymes and so on.

We shall commence our study of the uses of proteins

in the body by considering the nature of the breakdown processes occurring in tissues ; we shall then proceed to show how the lost constituents of tissues are replaced from the amino-acids absorbed from the alimentary canal ; and, lastly, we shall describe the chemical changes suffered by the waste amino-acids of the tissues and the superfluous amino-acids of the food during their utilisation as fuel in the body.

To begin, then, with the use of the proteins—or rather of the amino-acids obtained from them—in the building up of the constituents of tissues. There are, of course, some cases in which an abundant supply of amino-acids is obviously required for the formation of new protein—for example, in the growing organism whose total amount of tissue protein is increasing. Then again, the convalescent requires amino-acids in order to rebuild the tissue proteins that were broken down during his illness. But even in the normal adult organism a certain amount of protein breakdown is continuously occurring, so that a certain constant supply of amino-acid is required to replace this. In order to understand the processes that constitute this aspect of the metabolism of proteins it is necessary to obtain an idea as to what is involved in this breakdown. The question is : “What is the mechanism, and what are the products of this never-ending breakdown of the tissue protein?” We make our first approach to its answer when we realise that in the complicated system protoplasm there are not only proteins, but also proteolytic enzymes. Now it will be seen that it will require a very nice balance of conditions so to adjust things, that the protoplasm shall not fall a prey to the protein-splitting enzymes it contains. A small disturbance of that balance, such as, perhaps, an

excessive production of acid as the result of over-activity, or of insufficient circulation, will alter the conditions so much that the proteins of the living cell may succumb to the activities of its own enzymes. This can be easily illustrated by keeping a piece of muscle under aseptic conditions in an incubator at body temperature for a few days. Although bacteria are excluded, the proteins of the muscle break down, under the influence of its own proteolytic enzymes, until, finally, there is left little more than a mixture of amino-acids. This process of spontaneous disintegration of an isolated tissue is termed **autolysis**. Bearing these facts in mind it is not surprising to find that the breakdown changes resulting from wear and tear in a tissue also consist in a hydrolysis of its proteins to their constituent amino-acids. In fact, the main changes undergone by the tissue protein during the wear and tear are different only as regards situation or position, and not essentially in kind, from those changes that occur in the alimentary canal during the digestion of food proteins.

In order to understand how the amino-acids that are thus lost from the protein molecules composing living protoplasm are replaced, we must trace the fate of those amino-acids which, as we saw in the preceding chapter, are absorbed from the alimentary canal during the digestion of protein food. In the blood stream they are carried to all the tissues of the body, and it is found that after the absorption of a protein meal each tissue loads itself up with a supply of amino-acids from the common stock in the blood. The percentage of these uncombined free amino-acids in the various tissues can be shown by the nitrous acid method of analysis (p. 23) to be notably increased at this time. This is expressed in the curves

given in Fig. 2, which shows the results of an experiment in which, for convenience, amino-acids were introduced into the blood of a starving animal by injection rather than by the slower process of absorption from the alimentary canal. It will be noticed that the liver takes up a very considerable amount of the injected amino-acids, but that owing to the rapidity of further changes

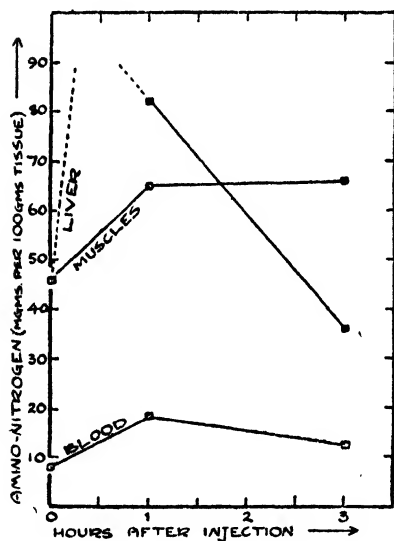


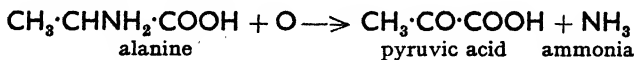
FIG. 2. Curves plotted from estimations by Van Slyke showing the extent to which amino-acids are taken up by various tissues of a fasting animal when these substances are injected into the blood stream.

The increased concentration of amino-acid nitrogen is most marked in the liver, but as the liver rapidly deaminates the amino-acids it receives, the exact maximum concentration in the organ is difficult to determine. On the other hand the amino-acids are retained by the muscles, and are used for the slow building up of their wasted substance.

that take place in this organ the increase of the amino-nitrogen concentration is only temporary. The muscles, on the other hand, retain more permanently the amino-acids they absorb—at least in a starved animal in which the tissues stand in need of amino-acids for the resynthesis of their lost substance. Doubtless after a protein meal the absorbed amino-acids are presented to the tissues

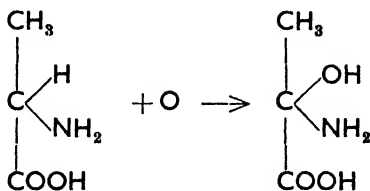
in the same sort of way, so that these may choose, as it were, the "building stones" they need. The rejected amino-acids—representing that portion of the protein food that is not required for more important uses than to be burnt as fuel—seem not to be dealt with further in the tissues, but being left in the blood, are ultimately carried round to and absorbed by the liver. This is how it happens that the liver accumulates amino-acids in such relatively large quantities—it receives all that the other organs have no use for.

In the liver the "fuel" amino-acids undergo a far-reaching decomposition in which their nitrogen—which, being non-combustible, is useless from the point of view of obtaining energy—is removed. This nitrogen is eliminated by the splitting off of the amino-groups as ammonia, the process being termed **deamination**. It is one of the most fundamental reactions in the whole of the chemistry of the body. The residual molecule now contains only carbon, hydrogen and oxygen, and so is suitable for complete combustion. As a matter of fact, a partial oxidation occurs at the same time as the removal of the amino-group in such a way that the non-nitrogenous residue from an amino-acid that has suffered deamination remains in the form of a **keto-acid**, that is, an acid in which the original position of the amino-group is now marked by the presence of the keto-group, :CO. If we take as an example, alanine (amino-propionic acid), then this on deamination gives ammonia and the particular keto-acid called **pyruvic**, thus :—

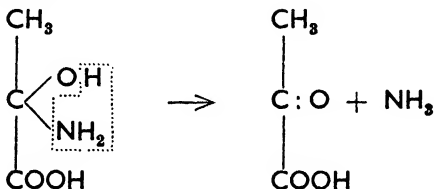


Of course even this apparently simple reaction takes

place in several stages. Most probably the oxygen atom first unites directly with the alanine to form a hydroxy-amino-acid :—

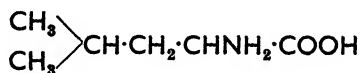


which then splits off ammonia :—



but this is by no means the only way in which the reaction could go. We shall have other possibilities to consider later (p. 62), but meanwhile we will point out that it is easy to show this decomposition of amino-acids by direct experiment, for if we perfuse a surviving liver with a solution containing amino-acids we find in the out-flowing liquid products from which the amino-group has been removed. The keto-acid itself is difficult to detect under these conditions, as in contact with the liver tissue it readily undergoes further changes. It is in many cases, for example, converted into sugar by processes that we shall describe in more detail later. But the great point at the moment is that non-nitrogenous, i.e. deaminated, products are formed from amino-acids in this way.

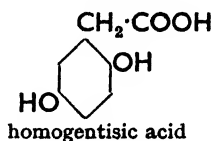
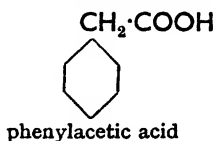
The importance of the liver in the metabolism of amino-acids has further been shown by observations on dogs in which this organ has been completely removed, without immediately killing the animal, by a skilful operation involving the preliminary short-circuiting of the hepatic blood circulation. Such an animal seems to have no power at all of metabolising amino-acids; when these are injected into the blood stream they do not rapidly disappear as would be the case in a normal animal, but remain unchanged in the blood and tissues and come over in large amounts into the urine formed during the period of survival. In a similar kind of way it is found that in cases of acute yellow atrophy of the liver in man, amino-acids are dealt with very incompletely, and some of them may come over into the urine in sufficient amount to crystallise out on standing. This is the case with **leucine**, a branched-chain amino-acid we have not yet met. It is α -amino-*isobutyl*acetic acid :



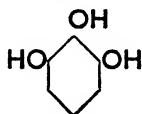
It is not surprising to find the relatively insoluble tyrosine also in the urinary deposit in this condition, while the abnormally high concentration of total amino-acids in the urine can be shown by the method of formol titration (p. 22).

Speaking of tyrosine and its behaviour during a condition when the normal line of chemical change in the body is departed from reminds us of a further very interesting way in which it has been proved that it is the keto-acid that is formed from an amino-acid during the process of deamination. In this case we take advantage

of the occurrence of a somewhat rare and apparently harmless disorder of metabolism known as **alkaptonuria**, in which the urine, when made alkaline, or allowed to become so on standing, absorbs oxygen from the air and turns black in colour (Arabic *al-kali* + Gr. *κάπτειν*, to gulp down). This behaviour is due to the presence in it of a substance called **homogentisic acid**, which is very easily oxidised in the air with the formation of dark-coloured products of oxidation. It will be seen that homogentisic acid is a di-hydroxy derivative of phenylacetic acid :—



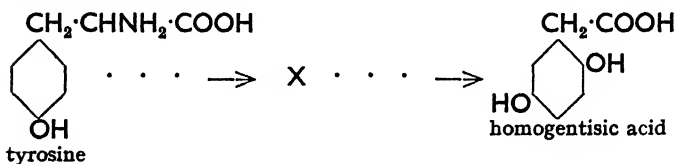
It is easy to understand why this substance should be so readily oxidisable. It will be observed that it contains two $\cdot\text{OH}$ groups attached to a benzene ring: and all hydroxy-derivatives of this ring are readily oxidisable substances. The most familiar example of this fact is furnished by pyrogallol, which contains three hydroxy-groups attached to the ring :—



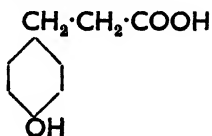
This is so easily oxidisable that in alkaline solution it readily absorbs oxygen from the air, forming dark-coloured products, and is therefore extensively employed as an absorbent for oxygen in gas analysis. Similarly it is only when homogentisic acid is in alkaline solution that it absorbs oxygen so that the urine of an alkaptonuric

patient turns black on exposure to air only after it has stood for some time so that it has become alkaline by the bacterial formation of ammonia from the urea.

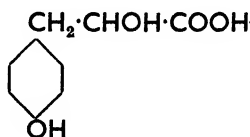
Now it is known for certain that homogentisic acid arises in the alkaptonuric patient from tyrosine, because if we withhold tyrosine from the diet the amount of homogentisic acid is reduced, and further, any tyrosine administered with the food gives rise to a corresponding extra amount of homogentisic acid in the urine. We can say, then, that tyrosine in the alkaptonuric patient gives rise to homogentisic acid through certain intermediate stages thus:—



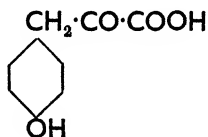
and we want to know what are these intermediate substances which we have called X. To do this we feed to the patient a few likely substances and see what happens. It is evident that if we hit upon a true intermediate stage between tyrosine and homogentisic acid, then we should expect this to give rise to homogentisic acid just as tyrosine itself does. But if we choose a substance which does not occur on the line of metabolism, we should expect to find it excreted either unchanged, or in some form other than homogentisic acid. Actually we find that if we feed the saturated acid



corresponding to tyrosine, it does not cause an increased output of homogentisic acid in the urine ; neither is the corresponding hydroxy-acid



converted to homogentisic acid during its passage through the body of an alkaptonuric ; but if we give the corresponding keto-acid,

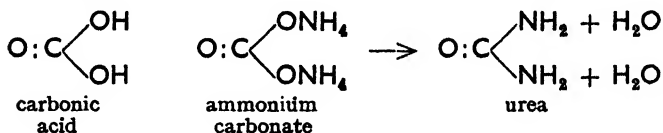


we find that it is quantitatively converted into homogentisic acid before it is excreted. So that we conclude that the keto-acid is an intermediate stage in the metabolism of tyrosine in the patient. It is possible that in the normal body tyrosine similarly first gives rise through the keto-acid to homogentisic acid, but that here the change goes further, the homogentisic acid being oxidised right down to carbon dioxide and water. The difference between an alkaptonuric patient and a normal person would then be that in the patient some enzyme necessary for breaking up this homogentisic acid is lacking.

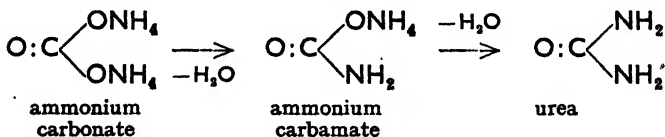
This is just one example of a very general method of investigation in which we obtain information as to the normal course of metabolism of some substance from observations on patients in whom that particular line of metabolism is deranged.

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We must now go on to a consideration of the further history of the ammonia and the keto-acids that we have thus shown to be formed as a result of the deamination of the amino-acids. Let us deal with the ammonia first.— This is liberated in the liver tissue where there is an abundant supply of carbonic acid with which it combines, forming **ammonium carbonate**. Now as long ago as the "eighties" of last century it was shown that when ammonium carbonate is perfused through the liver it is converted into **urea** by a process that has for long been regarded as a simple dehydrative splitting off of two molecules of water :—



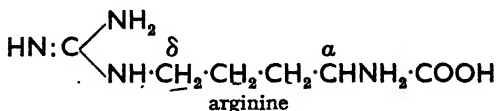
It has generally been supposed that this change occurs in two stages so that ammonium **carbamate** formed by the removal of only one molecule of water from the carbonate would form an intermediate step :—



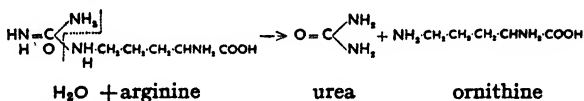
But for a quarter of a century or more it has also been known that the livers of mammals contain an enzyme capable of liberating urea directly by hydrolysis (without the intermediate liberation of ammonia) from a particular **amino-acid** known as **arginine**. As we have not yet mentioned this amino-acid we must first explain its constitution. It is a derivative of the 5-carbon member

of the fatty acid series, namely valeric acid, containing in the α -position the usual amino-group and in the δ -position at the other end of the chain a guanidine group

$\text{HN}:\text{C} \begin{array}{l} \text{NH} \\ \text{NH}_2 \end{array}$ which differs from urea only in the possession of a divalent $:\text{NH}$ (imino-) group in the place of the oxygen atom. The formula for arginine will therefore be written :—



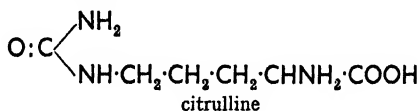
Now when this arginine is acted upon by its appropriate enzyme arginase, urea is, as we have said, hydrolysed off and a diamino-acid known as **ornithine** remains :—



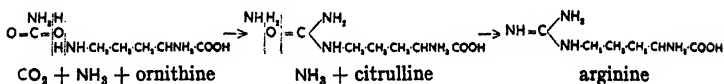
But this as it stands would account for the production of urea from one special amino-acid, namely arginine, only, and not from amino-acids in general. Quite recently, however, it has been discovered that this diamino-acid **ornithine** acts as a powerful catalyst in the production of urea by slices of liver tissue supplied with ammonia and carbonic acid ; from which it would appear that ornithine is an important intermediate product in the formation of urea from ammonium compounds. It must combine with ammonia and carbon dioxide to give some substance that in turn breaks down with the formation of urea and the re-liberation of ornithine, and if we suppose that this substance is arginine itself we at once see a very special importance in the occurrence of the enzyme arginase in

the liver for the production of urea, not only from arginine itself, but, via ammonia, from amino-acids generally. It is very significant in this connection that in birds, whose main nitrogenous end-product of metabolism is not urea but uric acid (p. 117), the liver does not contain much of the enzyme arginase.

So far, then, we have come to the conclusion that ammonia and carbon dioxide unite with ornithine in this way giving arginine which is then decomposed under the influence of arginase to give urea and ornithine which can thus be used over and over again. There is just one further point we should mention in connection with this formation of arginine from ornithine—it seems to take place in two stages, the first product being a substance called **citrulline**, whose constitution is similar to that of arginine except that it contains a urea residue in place of the guanidine thus:—



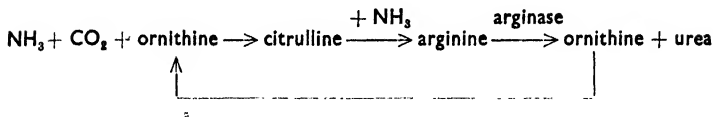
This substance derives its name from the fact that it was first found in the big green vulgar water-melon—*Citrullus vulgaris*. It is evidently formed by the union of 1 molecule of ammonia and 1 of carbon dioxide with ornithine, and is converted further into arginine by reaction with a second molecule of ammonia:—



The occurrence of citrulline in this way as an intermediate product is deduced from the observation that in contact

with liver tissue it is very rapidly converted into urea.

The whole cyclic process may be represented thus :—



We thus see that the disposal of the ammonia liberated by deamination involves a more complicated process than the simple dehydration hinted at above, but that, nevertheless, the end-result is the same, namely its condensation with carbon dioxide to yield the neutral, soluble and easily-eliminated substance urea, which constitutes, in mammals, all but a small fraction of the total end-products of the metabolism of nitrogen-containing substances, and, as such, in ourselves is excreted to the amount of about 30 grms. a day.

That this process of conversion of ammonia into urea is brought about particularly by the liver has been shown by trying with slices of other organs experiments similar to those just described. This was, however, originally proved by means of animal experiments in which the blood circulation through the liver was much reduced, although not completely abolished, by joining the portal vein directly to the inferior vena cava—an operation known as the establishment of an Eck's fistula—and has now also been confirmed by the more drastic method of complete removal of the liver. An animal with an Eck's fistula remains practically normal as long as it receives only carbohydrate food, but protein food makes it ill, probably because it is crippled, so far as its power of utilising amino-acids is concerned. The completely liverless dog is found to have no power at all of forming new urea, and as the amount of this substance already present in the

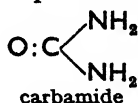
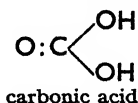
body is secreted in the urine the concentration of urea in the blood and tissues steadily falls.

The question of the behaviour of urea in these liverless animals is closely bound up with that of the ammonia. It is found that removal of the liver does not appreciably reduce the daily output of ammonia in the urine, so that the ammonia of the urine evidently does not arise from the liver; it is therefore not ammonia that has been left over from deamination and not converted into urea—in fact the conversion of ammonia to urea in the liver seems to be remarkably complete. Furthermore, there is only a slight increase in the concentration of ammonia in the blood and tissues, showing that the tissues of the body generally do not produce much ammonia, and when both liver and kidneys are removed even this slight increase does not occur. These observations support the view that the ammonia of the urine is formed in the kidney itself, and indeed it has now been shown that slices of kidney can deaminate amino-acids into ammonia and keto-acids—in some cases (e.g. the rat) more quickly, even, than an equal weight of liver tissue. From this it would seem that the “acidosis” that is associated with kidney disease is due to the inability of the damaged kidney to provide sufficient ammonia for the neutralisation of the acid end-products of the body's metabolism.

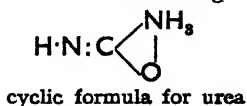
While we are discussing the formation of urea we might just mention that the reverse reaction, namely the formation of ammonium carbonate from urea, is well known. It occurs, for example, when urine is allowed to stand; under the influence of a ferment **urease** contained in the bacteria which develop in the liquid the urea is converted into ammonium carbonate and the urine becomes ammoniacal and alkaline. This very reaction is also made

use of in the most modern method for estimating the percentage of urea in urine and blood. In this case the necessary urease is obtained by making an extract of the soya bean, in which the enzyme is abundant. By means of this the urea is converted into carbon dioxide and ammonia, which latter is estimated by absorption in a measured volume of standard acid. Each molecule of urea gives two molecules of ammonia, so that from the quantity of ammonia formed the amount of urea can be readily calculated. For the purpose of a "urea concentration test," however, i.e. for following the rate at which urea is excreted after a dose of 15 gm. of it by the mouth the old inexact hypobromite method, in which the urea is estimated by the volume of nitrogen liberated when the urine is treated with sodium hypobromite, is usually sufficiently exact, and it has the advantages of simplicity and speed.

To return from this digression—our account of this subject of the relationships of urea and ammonium salts in the body would be incomplete if we failed to mention that in 1923 Werner published a monograph embodying the results of a decade of experimental work on the chemistry of urea, which has led him to the conclusion that the constitutional formula ordinarily assigned to urea is incorrect and that urea is *not* the amide of carbonic acid as we have already represented it:

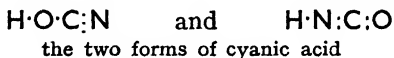


but is a ring compound of the following molecular structure:

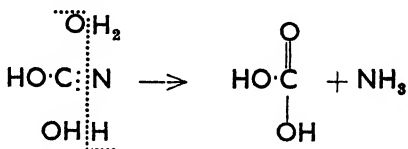


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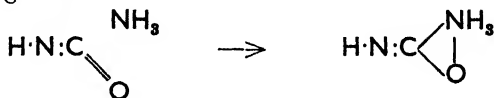
Further, Werner supposes that urea is formed in the animal body not by the dehydration of ammonium carbonate in the way already described, but by the decomposition of cyanic acid. The cyanic acid which on this theory is the precursor of urea in the body is supposed to arise directly from the oxidation of proteins. The mode of its conversion into urea is explained in this way:—Cyanic acid is capable of existing in two forms, so that its solution contains molecules of two kinds :



Now of these, the first form is readily decomposed by water into carbonic acid and ammonia, thus :



then the second form of the acid unites with this ammonia, producing urea :



the nitrogen atom originally present in the ammonia becoming, as nitrogen atoms often do, pentavalent.

Thus, on this view, the mode of production of urea in the animal body is similar fundamentally to the process discovered by Wöhler when, in 1828, he showed that when ammonium cyanate is gently heated it is converted into urea, a substance that until then had been obtained only from animal sources, and from this deduced the far-reaching generalisation that the constituents of living

organisms are not endowed through the operation of any indefinable "vital force" with special properties peculiar to themselves, but exhibit decompositions and syntheses governed by just the same definite laws as are found to apply in the inorganic world.

But although there is evidence that cyanic acid is produced as an intermediate product in the *decomposition* of urea under the influence of urease, and that the ammonia formed from the urea during this reaction arises immediately from the hydrolysis of the cyanic acid, attempts to obtain a *formation* of urea in solutions of ammonium cyanate brought into contact with liver tissue have failed—which would make it seem unlikely that any urea is formed in the body in this way.

And in any case in Werner's view of the constitution of urea there is a serious difficulty, inasmuch as the cyclic formula represents one of the nitrogen atoms as pentavalent. Now urea is not an electrolyte, i.e. in solution it is not dissociated into ions, so that all of these five valencies of the nitrogen atom would be non-ionising or "co"-valencies. But the modern study of atomic structure has shown that the nitrogen atom possesses five valency electrons: it can therefore share only three more electrons from other atoms, and so can make only three non-ionising or co-valent bonds, as in NH_3 , before making up its complement of eight electrons (which is the maximum stable number for any atom). In compounds where nitrogen is pentavalent it has always given away entirely one electron to another atom, thus forming an "ionising" or "electro"-valency: only then can it share one more electron from another atom and form one more (i.e. a fourth) non-ionising valency. Think, for example, of the four non-ionising

valencies uniting the hydrogen atoms to the nitrogen atom in ammonium chloride, and contrast them with the ionising valency by means of which the chlorine atom is held. It is these four non-ionising valencies that give the ammonium ion its stability, and the ionising valency by which the chlorine atom is anchored that confers upon it its property of ionising in solution. But the electronic structure of the nitrogen atom does not permit of the formation of *five* non-ionising valencies such as Werner's cyclic formula for urea would demand. We have here an illustration of the general principle that in assigning a chemical structure to a substance it is necessary not only to assign the correct *number* of valency bonds to each atom, but also to ensure by reference to our modern knowledge of atomic structure that the particular *types* of linkage are capable of being formed.

However, it would probably be admitted by most organic chemists that neither the carbamide nor indeed any other single formula adequately expresses the behaviour of urea, and that the substance as we know it probably consists of a mixture of at least two mutually interconvertible forms in which the arrangements of the atoms in the molecule are not the same.

But these considerations are taking us far beyond our intended scope. We have dealt at considerable length with the formation of urea and its relation to ammonia, because these substances are of such fundamental importance in the body, but we must now say something more about the keto-acids that form the other products of deamination.

The keto-acids are important substances because they represent, so to speak, the crossing-point of two main lines of metabolism—that of carbohydrates and that of

proteins. We have said that the proteins give us amino-acids. These on deamination give rise to keto-acids, and these are oxidised finally to carbon dioxide and water. Suppose, however, that the immediate oxidation of the keto-acids is not necessary for the body, then they are stored—and they are stored in the form of carbohydrate. The chains of the keto-acids are linked up to form glucose, and from this we readily obtain **glycogen** (p. 185) in the liver or other tissues. In the case of alanine the transformation is easy, for in its molecule alanine already contains a chain of three carbon atoms; we need to take only two such molecules, then, in order to give us the six-carbon chain of glucose.

In the case of amino-acids containing a four-carbon chain it is found that only three of the carbon atoms are utilised for the production of glucose, the other being oxidised away; and in a similar fashion from a five-carbon chain two atoms are removed before the three-carbon unit necessary for the formation of glucose is obtained. It is interesting to find that the two-carbon amino-acid glycine also gives rise to sugar, apparently by the deamination and coupling up of three molecules, but the process is certainly not so simple as at first sight it appears. On the other hand, there are some amino-acids, conspicuous among them being tyrosine and tryptophane, and in general all the amino-acids containing ring structures, that do not give rise to glucose at all. It will have been noticed that we have not mentioned the methods by which the sugar-forming capacities of the amino-acids have been investigated; the reason is that they involve the use of diabetic animals and so will be more readily understood when we come to deal with that derangement of metabolism (p. 207).

So far we have dealt with the amino-acids absorbed from the alimentary canal and destined to be used as fuel in the body. But we should mention that a similar process of deamination is the fate meted out to those amino-acids that become split off from the tissue proteins during the processes of wear and tear. Their nitrogen is removed as ammonia and the keto-acids remaining are converted into sugar and glycogen, and ultimately used as fuel. So that the urea of the urine is derived from two main sources—on an ordinary diet the greater part of it is derived from the nitrogen of the food proteins that are subserving no more important function in the body than to be used as fuel; this urea is formed from amino-acids that have never formed constituents of the living tissues of the body; it has been produced by the tissues, but not from their substance. Such a product of metabolism is said to be **exogenous** in origin. On the other hand, a certain amount of the urea of the urine is derived, as we have seen, from amino-acids which have been liberated during the breakdown of the living material itself. This portion of the urea is referred to as **endogenous**, for it has been formed from the inner structure of the protoplasm itself.

This endogenous urea may be compared to the rust which is formed on the iron-work of a machine, and which, representing, as it does, material lost from the actual fabric of the machine, must sooner or later be replaced if the machine is to maintain its working powers. An ordinary machine, however, differs from the body in the respect that while its breakdown product is non-combustible, the amino-acids that become split off from the body proteins can serve as fuel to the remaining tissues. It is the nitrogen of such amino-acids that goes to form the endogenous urea.

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CHAPTER V

THE METABOLISM OF PROTEINS (*contd.*): THE PRODUCTS OF TISSUE BREAKDOWN: CREATININE: NEUTRAL SULPHUR

"I would therefore call the protein metabolism which tends to be constant, *tissue* metabolism, or *endogenous* metabolism, and the other, the variable protein metabolism, I would call the *exogenous* or intermediate metabolism."—*Folin*.

IN the last chapter we saw that the chief nitrogenous product of the breakdown of tissue proteins is urea. But we found that urea is also a product of the utilisation as fuel of the excess amino-acids of the food. The amount of urea excreted in the urine per day will therefore vary not only according to the rate of tissue breakdown, but also with the amount of protein food eaten; and in general it will not be possible to decide what proportion of the total urea excreted is of exogenous and what of endogenous origin. But careful analyses of urine have revealed the fact that there are urinary constituents that are excreted in constant amount per day no matter how rich or how poor in protein the diet may be. Such constituents obviously cannot arise directly from the food or they would be formed in amounts determined by the proportions of the various food constituents eaten; they must therefore represent the end-products of processes taking place in the constituents of the tissues themselves, i.e. they must be entirely endogenous in origin, and as such must by their amount give a measure of the rate of tissue breakdown occurring in the body. It is with such products that we are about to deal

in the present chapter. It will be realised that it is a very important matter indeed to obtain some kind of measure of the amount of endogenous metabolism going on in a subject—a patient for example—because this represents the rate at which the tissues are breaking down; and evidently one must know at what rate the tissues are breaking down if the patient is to be dieted suitably.

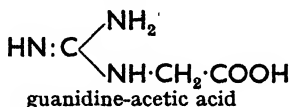
From what we have said it will not be difficult to understand how such a product of endogenous metabolism is to be recognised. What we have to do is to vary the amount of protein in the diet of an individual, and to determine which substances he excretes in constant amount, in spite of the changes in his food. This has been done by Folin, and he has discovered that the chief urinary constituent that shows this constancy of output on different diets is **creatinine**. Indeed, the daily output of this substance is so remarkably constant that it is used as a control for the completeness of collection of a 24 hours' specimen of urine; the actual constant quantity excreted being roughly proportional to the total amount of muscular tissue in the particular subject's body. For example, on a diet poor in proteins one of the subjects investigated by Folin produced 1.6 grm. of creatinine per day, and on a diet rich in protein he gave out a practically unaltered quantity of this substance, namely, 1.55 grm. The contrast between this constancy of production of creatinine and the variability of the output of urea is remarkable. On the diet poor in protein the same individual excreted 4.7 grm. of urea a day, while the amount was increased to 31.6 grm. when he received the richer food. These facts serve to mark off creatinine quite definitely as a truly endogenous product. That it is a true final end-product of metabolism is shown by the fact that when administered by the mouth creatinine

is excreted unchanged in the urine—a circumstance that does not in any way affect the value of the creatinine output as a measure of endogenous metabolism because creatinine is not present in any ordinary articles of diet.

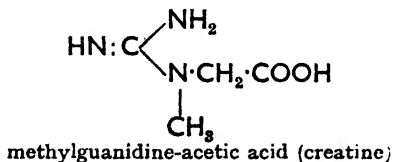
In order to understand the further relationships of this substance we must refer in outline to its chemistry. Creatinine is a ring compound whose constitution is most easily understood by considering that of related simpler substances. To begin with, we must remind the student that by replacing the divalent oxygen atom in the molecule of urea by the divalent imino-group, :NH , we obtain a basic substance composed only of carbon, hydrogen and nitrogen, known as guanidine:—



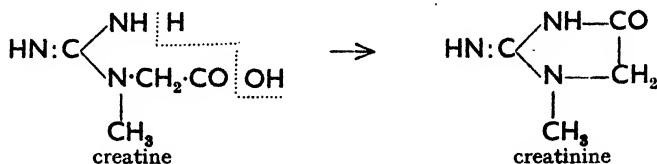
This guanidine readily enters into the composition of more complicated molecules, for example, guanidine-acetic acid is one of its more important derivatives:—



The substitution of a methyl group in place of the remaining hydrogen atom of the original $\cdot\text{NH}_2$ group involved in the condensation gives us methylguanidine-acetic acid or **creatinine**:—



a substance which we shall have to mention again later. Creatinine is the internal anhydride of this:—



One circumstance that renders creatinine so valuable as a measure of the rate of endogenous metabolism is the ease with which it is estimated. In order to determine the amount of creatinine in a sample of urine all that is necessary is to add some caustic soda and picric acid, when an orange-yellow coloration due to the formation of a creatinine picrate is produced. The depth of this colour is compared by means of a colorimeter with that given by a standard solution of creatinine; then, since the intensity of colour produced is proportional to the amount of creatinine, other things being equal, all the data are available for calculating the percentage of creatinine in the urine under examination.

It is no easy matter to make out the steps involved in the chemical changes that occur in the formation of the products of tissue metabolism, and the case of creatinine forms no exception—as witness the many varied views that have been put forward from time to time on the subject. All the muscles of the body contain very considerable quantities of the previously-mentioned closely-related substance **creatine** (Gr. *κρέας* = flesh, muscle) which must be very carefully distinguished from **creatinine** itself. This ending *-ine* means “derived from” and creatinine can, in fact, be so easily derived from creatine—by boiling with a little strong acid or even by

merely evaporating its solution—that, it is only natural to suppose that they are related metabolically as well. But this being the case there at once arises the question as to which of these substances gives rise to the other—as to which, in other words, is to be regarded as the raw material and which the end-product of this particular line of metabolism. At first it was thought that creatinine was formed at a constant rate in the body, and is converted into the creatine which is stored in the muscles. Then, when these are “saturated” with creatine, all further quantities of creatinine produced are excreted unchanged in the urine. But since then it has been pointed out that there is a very close parallelism between the creatine content of the muscles of an animal and the rate at which it excretes creatinine in its urine; and this observation has given rise to a tendency to regard the creatine of the muscles as being the primary product and the precursor of the creatinine of the urine. At first it was difficult to reconcile this idea with the observation that the casual administration of creatine by the mouth gave rise to no significant increase in the output of creatinine in the urine. But more recently it has been realised that the muscles constitute a remarkably effective “reservoir” for creatine—a reservoir which, moreover, is ordinarily not completely filled, so that the whole of a single dose of creatine would seem to be easily taken up in the muscles and so would not show itself by any increase in the output of a derived product. And now it has been established that if creatine is administered not in a single isolated dose, but continuously over a period of some three weeks, then all the creatine reservoirs of the muscles become filled, as it were, and from then on the continued administration of creatine *does* give rise to a correspondingly increased output of creatinine, both in man

and other animals. It is, therefore, nowadays believed that the creatine of the tissues is the precursor of the creatinine of the urine. It would be only natural to look for an enzyme responsible for this conversion of creatine to creatinine in the body, but so far none has been definitely shown to exist. Seeing, however, how easily creatine is dehydrated to creatinine in the laboratory it would seem that under body conditions creatinine would be slowly formed at a constant rate from the stored creatine of the muscles without the aid of any catalyst at all, and this idea is supported by the observation that, unlike so many of these metabolic reactions, the conversion of creatine into creatinine is not localised in the liver; for complete removal of this organ does not affect the creatinine output during the period of survival.

As, then, we suppose that the creatinine of the urine arises from the creatine of the muscles it is of interest to enquire whether muscular activity has any influence on the output of these substances. The idea was at one time put forward that this conversion of creatine into creatinine was associated with the maintenance of that condition of continuous contraction known as "muscle tone"; for the rate of excretion of creatinine by a man standing "at attention" was said to be greater than when he was carrying out gymnastic movements. But while the experiments on which this idea was based are not now regarded as adequate to prove the point, it is clear that during a period of muscular activity the creatinine output is somewhat greater than during a period of rest. But after the exercise there follows a period of diminished creatinine excretion, so that in the long run over a period of 24 hours the total output of creatinine comes to be the same whether exercise is indulged in or not. It would seem as if the "wear and

tear" of the muscular machine is the greater the harder it is driven, but that it is recuperated during its periods of rest.

Of course, in deriving as we have, the urinary creatinine from the creatine of the tissues we have explained only the final step in a probably complicated series of changes, for we have said nothing about the origin of the tissue creatine itself. As a matter of fact, we are still quite uncertain as to how this is formed. As we have explained, creatine is a methyl derivative, and as we eat so few substances—apart from the muscle creatine that we eat in meat—that are methyl derivatives, it seems certain that this methyl group must be introduced by some process in the tissues. That the body certainly can introduce methyl groups into compounds is not doubted—for example, pyridine taken by the mouth is excreted in part as methylpyridine!—but the details of the process are at present obscure.

With regard to the physiology of creatine itself we shall have much more to say when we come to consider the chemistry of muscular contraction. Suffice it to say at the moment that it itself appears in the urine under certain conditions, such as, for example, during starvation and during the involution of the uterus after pregnancy, when a considerable amount of muscular tissue is breaking down and its creatine is being liberated in abnormally large quantities into the blood. Creatine is also a normal constituent of the urine of children of both sexes, and it is liable to occur periodically in the urine of normal adult women. But it is never present in the urine of normal adult males.

Leaving these substances we now pass on to notice that among the sulphur-containing constituents of the urine there are some whose output is not affected by changes in

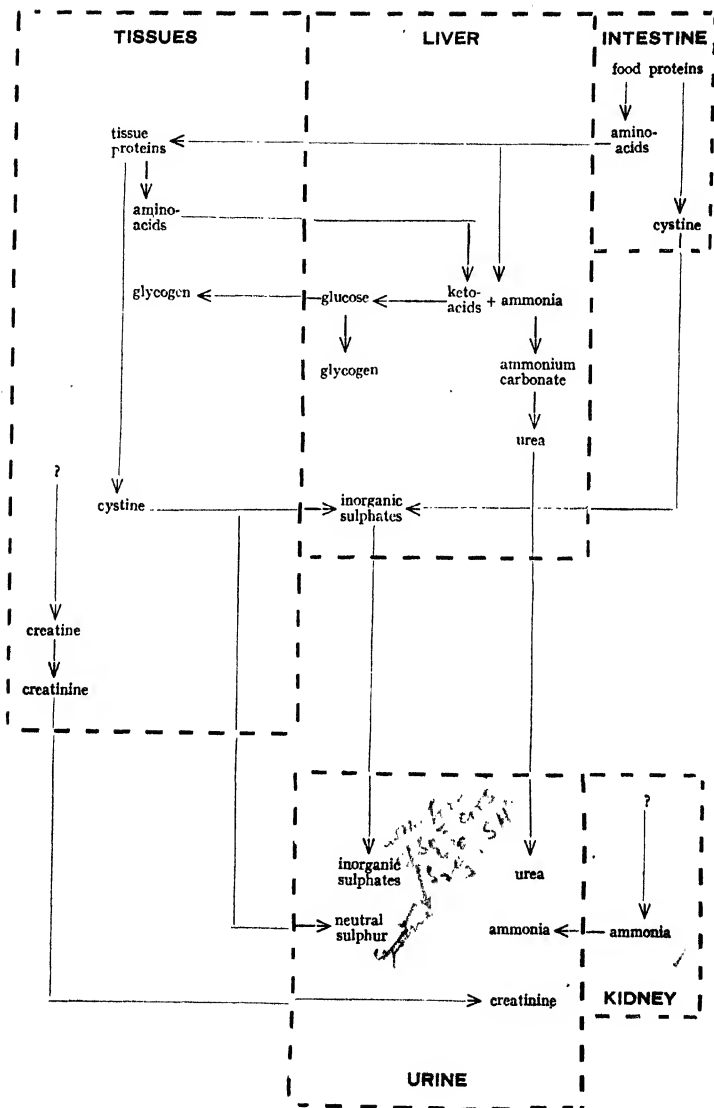
the composition of the food, and which, therefore, also represent the endogenous end-products of tissue processes. Sulphur is taken into the body mainly in the form of the sulphur-containing amino-acids cystine and methionine (p. 20). Any cystine that is absorbed from the alimentary canal in excess of the requirements of the tissues seems to be quickly oxidised in the liver in such a way that its sulphur is promptly excreted in the urine in the form of inorganic sulphates. But if we estimate by suitable processes of oxidation the total sulphur present in a given sample of urine we find that it is invariably greater than the amount of sulphur present as sulphates. In other words, the urine must contain sulphur compounds in which the sulphur is linked chemically not with oxygen (as it is in sulphuric acid and the sulphates), but with hydrogen, or (as in cystine itself) with carbon. Such sulphur, not forming a constituent of an acid or its salts, is called **neutral sulphur**, and in the urine it occurs as small quantities of a number of miscellaneous compounds including thiocyanates, thiosulphates, mercaptan (p. 19) and diethyl sulphide $C_2H_5 \cdot S \cdot C_2H_5$, the sulphur analogue of ordinary ether. That all these together represent products of tissue metabolism is shown by the results of observations such as that of Folin quoted on page 78. In this metabolism experiment it was found that the amount of inorganic sulphate excreted by the subject varied with the amount of protein in his diet, being 3.3 grm. per day when the rich protein diet was taken and falling to less than 0.5 grm. on the poorer diet. In contrast with this the daily excretion of neutral sulphur remained virtually constant throughout, being 0.18 grm. on the rich and, if anything, slightly more — 0.20 grm. — on the poorer diet. The rate of excretion of the compounds included under the term neutral sulphur

gives us, therefore, a measure of the rate at which certain tissue processes are occurring, but in clinical work this fact finds but little application on account of the relatively complicated procedures required for the estimations involved. The estimation of creatinine is of far greater importance, for as we have seen it can be carried out by a simple colorimetric method that possesses the great advantage of rapidity—a valuable asset in any method used for the investigation of life processes.

Before concluding this account of the occurrence of sulphur compounds in the urine we should just mention that there exists a rare disturbance of metabolism known as **cystinuria** in which the body seems to have lost the power of utilising the cystine of proteins, so that this amino-acid is excreted in considerable amounts in the urine and may even crystallise out in the form of urinary deposits or calculi. The curious feature of this condition is that although the cystine combined in a protein is but poorly utilised the patient can metabolise free cystine almost completely—even that which he himself has previously excreted!

SUMMARY OF THE CHIEF CHEMICAL CHANGES THAT PROTEINS UNDERGO IN THE BODY

The processes described in the last three chapters can be very conveniently summarised by means of the following diagram. It should be pointed out, however, that this diagram is merely one of several possible ways in which the outlines of the metabolism of proteins may be expressed; and that the student will derive full benefit from his reading of this subject only if he will work out his own schematic summary step by step as he follows the argument.



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The fundamental paper by

FOLIN, O.: "*A theory of Protein Metabolism*" (*Amer. Journ. Physiol.*, vol. 13 (1905), p. 117).

should be read by all students of biochemistry.

With regard to creatine and creatinine—although we have dealt with them in barest outline, as is appropriate in an introductory volume, yet there is an interesting and extensive literature dealing with the puzzling relationships of these substances. Fortunately this has now been summarised and critically reviewed in a monograph entitled:

HUNTER, A.: *Creatine and Creatinine*. (Monographs on Biochemistry; London: Longmans, Green & Co.)

For a full account of the neutral sulphur compounds of the urine see

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and

KAHN, M., and GOODRIDGE, F. G.: *Sulfur Metabolism* (New York: Lea and Febiger).

Further information regarding the condition of Cystinuria is to be found in GARROD's book referred to in the bibliography to Chapter IV, and in

LEWIS, H. B.: "*Sulphur Metabolism*" (*Physiological Reviews*, vol. 4 (1924), p. 394).

Some very recent observations on Cystinuria, together with a discussion of Sulphur Metabolism, are to be found in

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CHAPTER VI

THE NITROGEN EXCHANGES OF THE BODY AS A WHOLE : NITROGENOUS EQUILIBRIUM : PROTEIN STARVATION : THE USES OF PARTICULAR AMINO-ACIDS

"Enough is as good as a feast."—*Old Proverb.*

IN the preceding chapters we have considered the chemical properties of proteins and amino-acids, and the *details* of the changes which these undergo in the body. But long before we had acquired as much detailed knowledge as we possess to-day of the chemical relationships of the proteins attention had already been directed to the *general* uses of proteins in the body, and to the question as to the quantity of protein that an adequate diet should contain. Old, then, as these topics are, we have deferred until now our main consideration of them because they are so much more intelligible in the light of the newer information we have already given. We have, however, already mentioned in passing that the most important use of proteins in the body is for the replacement of the materials lost during the wear and tear of the tissues, and that any quantity of protein taken in excess of that required for this purpose is decomposed in such a way that its carbon and hydrogen are ultimately used as fuel, while its nitrogen is got rid of—is wasted—in the urine. This was clearly realised by Voit as long ago as 1862, when he showed that during the day following the feeding of a kilogram and a half of meat to a dog an amount of nitrogen exactly equal to that contained in the meat was eliminated from the body of the animal. Most of this excreted nitrogen was

present in the urine; only a little of it was found in the faeces. On the other hand, the amounts of carbon and hydrogen leaving the body during the same day as carbon dioxide and water, in urine, expired air and faeces were less than those contained in food. A considerable portion of these energy-producing elements had evidently been retained in the dog's body in the form of non-nitrogenous molecules.

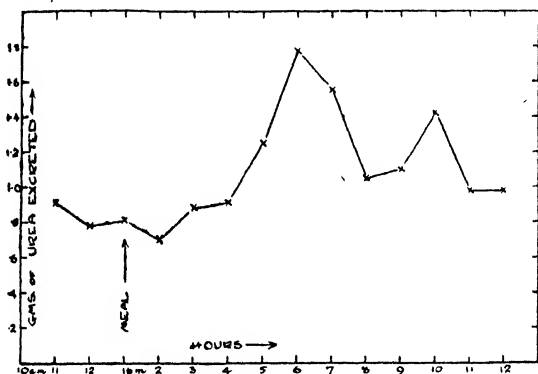


FIG. 3. The hourly excretion of nitrogen as urea in the urine during the period following a single protein meal.

To illustrate the promptness with which the body eliminates excess of nitrogen not required for repair of tissues (from Hopkins).

At a later date the same was shown to be true of the human subject by Hopkins, who followed the excretion of urea in the urine during the period following a protein meal. The results of one of his experiments are plotted in Fig. 3, which shows the hourly excretion of urea in the urine of a human subject after a single meal composed of 250 grms. of beef and 100 grms. of bread, following a fast of eighteen hours. It is seen that the urea excretion increases very soon after the taking of the meal, that it

follows a variable course showing several maxima, and that it has returned almost to the fasting value about ten hours after the food was eaten. Naturally under more usual circumstances, when food is taken at frequent intervals, the increase in the rate of output of urea following any one meal is masked to a great extent, because the nitrogen of the successive meals is eliminated in part simultaneously.

But when the meals are taken not in obedience to the dictates of appetite and custom, but at the intervals demanded by the scientific investigation it is easy to show that, in man also, during the 9 or 10 hours following a single protein meal an amount of nitrogen practically equal to that contained in the meal is excreted in the urine. And this occurs with the same promptness when the subject is at rest as when he is carrying out muscular work—an important point to which we shall have to refer again when we are discussing the chemical mechanism of muscular contraction (p. 254), for it shows that the deamination of the amino-acids goes on independently of the use of their carbon and hydrogen as fuel. In other words, the body has practically no power of storing up nitrogenous substances in the same sort of way as it stores fats and carbohydrates; the nitrogen of any protein taken in excess of that required for the maintenance of the tissues is wasted.

These considerations lead naturally to the question as to what is the minimum amount of nitrogen, in the form of protein, which must be present in an individual's diet in order that there shall be sufficient of this essential element for the maintenance of his tissues, while at the same time there shall be as small an excess of protein as possible left over for the less essential function of providing fuel.

This is an enquiry that is important, apart from its value as a piece of scientific knowledge. It acquired considerable prominence during the war, when rationing schemes had to be devised in order to ensure an appropriate distribution of the diminishing supplies of nitrogenous food. Then also an answer to this question is important from a general sociological point of view. The nitrogenous foods—meat, for example—form the more expensive items of one's diet : it becomes, therefore, a very important thing to know whether the earnings of the poorer classes of any community are sufficient to enable them to buy at least the minimum amount of nitrogen, in the form of proteins, which they need in order to maintain their tissues. For if in any community it happens that the poorer classes cannot afford to buy such a sufficiency of nitrogenous food, then they will be living in a permanent condition of under-nutrition. As a result, the foundations of such a society are weakened, so that the whole structure becomes liable to collapse.

Now we can decide whether the tissues of a subject are wasting permanently, or whether they are being repaired as fast as they break down, by comparing the amount of nitrogen in his food with that which leaves his body in the urine and fæces. The difference between the amount of nitrogen taken in and that in the fæces represents the amount absorbed from the alimentary canal into the body. If no more nitrogen than this is excreted in the urine then the tissues cannot be losing permanently any nitrogen ; but if more of this element is excreted than is absorbed, then evidently some of the excreted nitrogen must have come from the tissues, and therefore the amount of living matter in the body is diminishing. When an individual is giving out in his urine an amount

of nitrogen equal to that which he is absorbing he is said to be in **nitrogenous equilibrium** : when this condition is satisfied we know that all the nitrogen lost from his tissues is being exactly replaced ; for seeing that his intake and output of nitrogen are exactly equal, the total amount of nitrogen in his body must be remaining constant. Naturally, although the nitrogen given out from the body under these circumstances is equal in amount to that which has just previously been absorbed, the actual nitrogen atoms concerned in the two cases are not identically the same. For part of the excreted nitrogen will have come from the tissues and to replace this endogenous portion an exactly equal quantity will have been retained from the food ; only the remainder of the food nitrogen will pass directly over into the urine. Of course a growing organism can never be in nitrogenous equilibrium, for it gives out less nitrogen than it receives, retaining the rest in the form of the new tissues which it is building up. But the adult, fully-grown organism cannot retain more nitrogen than is required to maintain his already existing tissues. He cannot produce entirely new tissue proteins merely by eating excess of protein food, neither, as we have seen, can he store nitrogen in any other form. It follows, therefore, that if a man is in nitrogenous equilibrium on a diet containing a certain amount of protein he will be necessarily in nitrogenous equilibrium on a diet containing more protein, for he will excrete all the nitrogen over and above the minimum amount which is necessary for the establishment of nitrogenous equilibrium. Naturally, also, a starving man cannot be in nitrogenous equilibrium, nor can a man who is receiving no protein food, for in each of these the breakdown of the tissues still occurs, so that endogenous nitrogen appears in the urine,

while no nitrogen is being taken in to replace what is being lost.

There have been a number of different estimates of the minimum quantity of protein food required per day by a normal man in order to maintain nitrogenous equilibrium. As time has gone on, the tendency has been to assess the amount at a smaller and smaller value. One of the most recent attempts to determine it was that in which a laboratory servant in the Nutrition Institute at Copenhagen was the victim. They fed this man for a period of several months on a diet composed only of potatoes and margarine flavoured with onion! His fuel requirements were adequately supplied by the fat and carbohydrate he received, and, as a matter of fact, the small percentage of protein present in the potatoes was found to be sufficient to maintain nitrogenous equilibrium. As a result of analysis it was found that the intake and output of nitrogen of this unfortunate fellow were only about 5 grm. a day, but medical examination at the end of the experiment failed to discover any departure from a condition of robust health. Proteins, on an average, contain about one-sixth of their weight of nitrogen, so that the daily consumption of protein in this case was about 30 grm., i.e. just over an ounce—an amount considerably less than is included in an average diet, chiefly because potatoes and margarine do not appeal to our English palate so powerfully as meat and eggs. The protein portion of one's diet is in general the most appetising.

These 5 grm. of nitrogen represent, then, the amount which must be supplied to a normal healthy man per day in order that the loss of nitrogen from his tissues shall be compensated. If he receives less

nitrogen than this, this loss nevertheless continues at an undiminished rate, so that his body wastes. If he receives no food at all—not even carbohydrate and fat—then the rate at which his tissues break down is much accelerated. For now the tissue proteins are called upon to supply the fuel material necessary for the carrying on of the more essential functions of the body, such as the heart-beat and the respiratory movements. While the non-nitrogenous fuel reserves—the glycogen of the liver and muscles, and the fat of the fat depôts—last, the extent to which the tissue proteins are drawn upon is small. The nitrogen output in the urine during the first stages of starvation is comparatively small ; it sinks below the normal value for the well-nourished animal because there is now no nitrogen from exogenous sources to be excreted. But as the fat and carbohydrate reserves tend to become depleted, the extent to which the tissue proteins are drawn upon gradually increases. They are hydrolysed and deaminated, and the resulting keto-acids are ultimately used as fuel, while the nitrogen is excreted as urea in the urine. As a result of the increased use of the tissue proteins for fuel the nitrogen output is also increased. This increase of nitrogen excretion usually precedes the death of the animal by only a few days. The body has but little protein that it can really spare for use as a fuel. When this little is exhausted, the animal has no fuel of any kind available for carrying on its life processes, and it dies. The lack of available protein is shown in this last stage of starvation by the rapid fall in nitrogen excretion. These facts are illustrated in the curve in Fig. 4, which is taken from the results of an old experiment by Falck on a lean dog.

It must not be supposed that during this utilisation of the tissue proteins for the supply of energy the various organs of the body contribute of their substance to equal extents. Indeed, it is found that the relative loss of weight which has been suffered by any particular organ by the time that an animal dies of starvation may be very

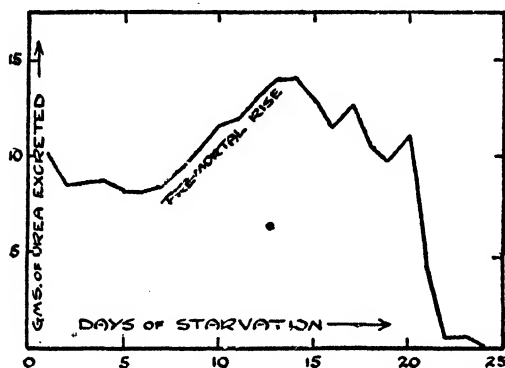


FIG. 4. The excretion of nitrogen as urea in the urine of a dog during three weeks' starvation.

At first, as a result of deprivation of protein food, the urea output falls to a low and more or less constant value. When the non-nitrogenous fuel reserves of the body are approaching exhaustion the animal begins to live at the expense of its own tissue proteins. This is marked by a rise in the amount of urea excreted, which reaches a maximum, and then falls off again when all the available protein has been utilised, and death is imminent. (Plotted from Falck's observations.)

different from that which has occurred in another. On the whole it is found that the least essential organs lose the greatest percentage of their respective weights, while the substance of the more vital organs is much more carefully conserved. For example, while adipose tissue may have disappeared almost entirely from the body and the stores of glycogen in the liver have been so far

exhausted as to have diminished the weight of that organ to half the normal value, while even voluntary muscles may have lost as much as a third of their original weight, the heart and brain will have given up a small fraction—a thirtieth or less—of their more valuable substance. There is much significance in this behaviour of the body to starvation, for it is of the utmost importance to a

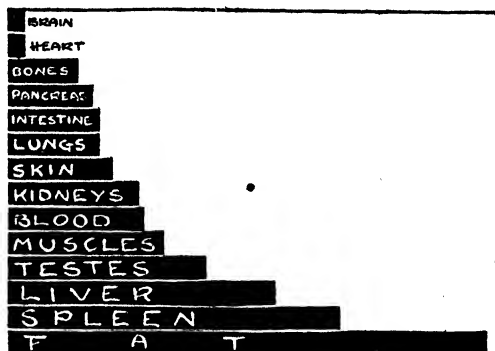


FIG. 5. A diagrammatic representation of the relative loss of weight of various organs during starvation as percentages of their initial weights.

Contrast the loss of 97 per cent. of the weight of the stored fat of the body with the diminution of only 3 per cent. in the weight of the brain; and the loss of 31 per cent. of the weight of the skeletal muscles with a loss of only 3 per cent. of the weight of the heart. (Constructed from Voit's data.)

starving animal that its heart-beat should be maintained at all costs in order that its brain may not be deprived of blood, so that the beast may remain alert and ready to take the fullest advantage of any opportunity that may offer of obtaining the food of which it stands in desperate need. The relative losses of weight of various organs during starvation are represented diagrammatically in Fig. 5.

So far we have spoken as if it were merely the total amount of protein nitrogen taken in per day which determined the condition of nitrogenous equilibrium. But if we allow our modern knowledge of the chemistry of the proteins to bear on this question it will be realised that besides dealing in this way merely with the total daily intake of nitrogen we must also bring into consideration the forms in which this nitrogen is combined—that is the nature of the amino-acids that compose the proteins eaten. For, as we have so abundantly illustrated, it is the amino-acid that is the significant unit so far as not only the chemistry but also the metabolism of proteins is concerned. Now the amino-acids are not in general interconvertible in the body. If any particular amino-acid is required for building up the tissues, then as a rule that particular amino-acid must be supplied ready formed in the food. If the tissue proteins are losing a certain amino-acid during their breakdown, such an amount of protein food must be supplied to the body as will contain sufficient of the particular amino-acid in question to replace that which is being lost, if nitrogenous equilibrium is to be maintained. For example, if histidine is being lost at a certain rate from the tissues, then for nitrogenous equilibrium the food proteins must contain sufficient histidine to replace this. Suppose that the food proteins contain, in addition, a large amount of some other amino-acid, say, glycine, which happens not to be required by the tissues, then the nitrogen of this excess of glycine must necessarily be wasted, since it is not in the required form. It follows, therefore, that a smaller amount of a protein that contains the various amino-acids in about the same proportions as those in which they are lost from the tissues will be required for the maintenance

of nitrogenous equilibrium than of one in which the amino-acids occur in other proportions. We also see that the rate of nitrogen output in the urine of a man who is receiving just the minimum amount of protein sufficient for maintaining his tissues—it was about 5 grm. in the case of the Copenhagen laboratory attendant—is not a true measure of the rate at which nitrogen is being lost from the tissues ; for the nitrogen of the urine under these conditions represents not only that which has come from the tissues, but also that of the food amino-acids which the tissues had no use for, and which therefore were used merely as fuel. This accounts for the observation that, in general, in order to maintain a man's tissues, it is not sufficient to supply him with the same amount of nitrogen in the food proteins as he excretes on a protein-free diet. It is necessary to supply a larger amount of protein than this, for usually a certain portion of some of the amino-acids will not be required by the tissues, and so will be useless for replacing their wear and tear.

These considerations supply the explanation for that prodigality of the body with regard to nitrogen which has long been realised. Without a knowledge of the needs of the tissues for particular amino-acids it would be surprising that even when the body is receiving less nitrogen than it requires for the maintenance of nitrogenous equilibrium, it does not retain all it receives, but excretes some in the urine. For if even a small amount of protein nitrogen be fed this leads to an increased output of nitrogenous substances even in a starving, wasting animal. If the amount of nitrogen in the diet is gradually increased, the difference between the amount given out and the amount taken in becomes smaller and smaller, so that the two curves representing nitrogen income and

nitrogen output respectively gradually approach each other until, when there is a certain amount of nitrogen in the diet, they coincide. This is the point of nitrogenous equilibrium (see Fig. 6). Any further increase of food nitrogen beyond this point leads to an exactly equal

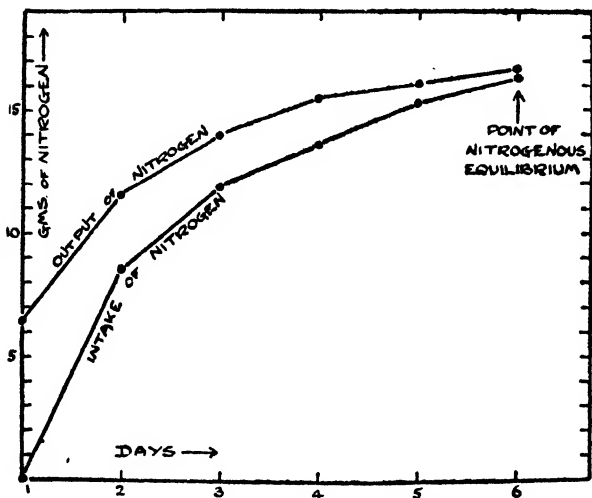


FIG. 6. Curves showing the gradual attainment of the condition of nitrogenous equilibrium on increasing the amount of protein fed to a dog.

Note that in order to attain the equilibrium it is necessary to give in the food a larger amount of nitrogen than is excreted during protein starvation because, in general, a portion of the food nitrogen will be in the form of amino-acids not required by the tissues. (Plotted from Voit's data.)

output of nitrogen, and so to a maintenance of the equilibrium.

If the reader has followed our account up to the present point he will probably have already realised that it constitutes a direct argument in favour of cannibalism.

100 FUNDAMENTALS OF BIOCHEMISTRY

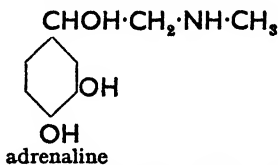
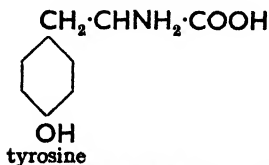
For in general it will be in the proteins of another member of its own species that an animal will be most likely to find the various amino-acids in the proportions required for building up its own tissues. And this has, indeed, proved to be the case, inasmuch as it has been found that a dog can be kept in nitrogenous equilibrium with a smaller total daily intake of nitrogen if fed on dog's flesh than if fed on the flesh of any other animal. Civilised man has, however, found a way round his repugnance at the idea of eating his fellow in order to maintain himself most economically in nitrogenous equilibrium: he simply eats a great variety of protein foods, and so ensures that he obtains each of the necessary amino-acids from one source or another. In doing so, however, he usually does not choose his proteins so scientifically as to reduce his daily protein intake to the 30 grm. that form the bare minimum consistent with the maintenance of his tissues. Most of us—merely as a matter of custom apparently—eat round about 100 grm. of protein a day, and dieticians in general have nothing to say against it. They merely stipulate that at least 30 grm. of the daily protein ration shall consist of high value proteins, i.e. proteins derived from animal sources such as meat, eggs, or milk. These animal proteins contain the amino-acids in more nearly the proportions in which we require them than do the somewhat more aberrant proteins of plants. The relative "biological values" of the various proteins from the point of view of their use for maintaining human tissues are expressed as the number of grams of human protein that could theoretically be obtained from 100 grm. of the protein under consideration. It is evident that judged by this standard the biological value of a protein that contains none of some amino-acid that is

required for tissue maintenance, and that cannot be synthesised from other substances in the body, must be 0, for no matter how much of it is eaten no complete human protein can be built up from the products of digestion. But if such an incomplete protein is taken together with a second protein, itself perhaps incomplete, but containing an abundance of the particular amino-acid lacking in the first, the biological value of the mixture may be very high. An interesting case of this kind is furnished by the proteins of milk. Of these casein contains but little cystine, and so has but a low biological value. But the other protein of milk—lactalbumin—contains, in common with albumins generally, a relatively high percentage of cystine, so that the naturally occurring mixture of these two proteins yields a complete set of the amino-acids we require. In other words, the biological value of the mixture is much greater than that of either constituent separately. Similarly the biological value of gelatin is zero on account of the absence of tryptophane and tyrosine from its molecule, but it is, nevertheless, a valuable source of other amino-acids when supplemented by other proteins that make up its deficiencies. So that in the end our study of the protein requirements of the body has served simply to emphasise the desirability of taking a "good mixed diet"—a prescription towards which very few of us feel any repugnance.

The question of the general use of proteins in the body thus involves a consideration of the uses of the individual amino-acids. It would be inappropriate, therefore, to conclude this account of the general metabolism of nitrogen without mentioning such definite information as we possess as to the functions of some of these substances. It is known, for example, that tyrosine and tryptophane

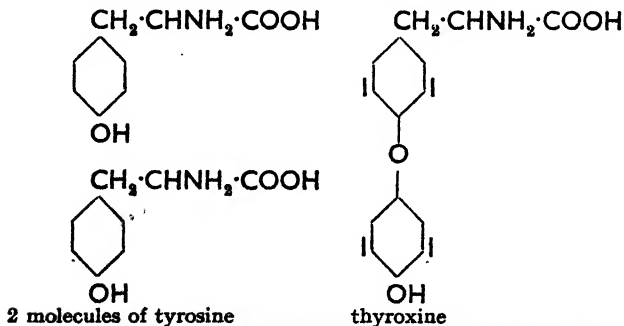
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are absolutely essential for animal life ; if these be omitted from an animal's diet it soon dies. It has been suggested that these amino-acids are necessary for the production of indispensable constituents of internal secretions, and there is no doubt that in the case of tyrosine this is true. We have not the space to go into details, but suffice it to say that it has long been known that **adrenaline**, the characteristic product of the medulla of the suprarenal glands, is closely related in chemical constitution to tyrosine as a comparison of the following formulæ will show:



(Note that adrenaline is a dihydroxyphenyl derivative of hydroxyethylmethylamine.)

and recently it has been shown that **thyroxine**—the active principle of the thyroid gland—is not, as was at one time supposed, chemically related to tryptophane, but is an iodine derivative that is to be regarded as arising from two molecules of tyrosine, thus :—



(Note that thyroxine possesses an "ether" linkage between the two rings, and contains 4 atoms of iodine.)

The extreme importance of tyrosine in the body can require no further emphasis than this.

The fact that tyrosine and tryptophane are essential to animal life indicates that the ring groupings they contain cannot be synthesised in the animal body; they must be supplied ready made. On the other hand, plant protoplasm has the power of elaborating these compounds from their elements, so that it is from food plants that all the tyrosine and tryptophane in the whole animal kingdom have been derived. Plants can make ring compounds while animals cannot. Contrasted with these we have such simple amino-acids as glycine, which have been shown to be readily synthesised from other substances in the animal body, and so need not be given ready formed in the food. Occupying a position, as it were, intermediate between these two groups of the indispensable and the non-essential amino-acids, we have a group of amino-acids which, while not necessary for the well-being of an adult organism, must be present in the food of a young animal if growth is to take place. An example of this class of amino-acids is lysine—the α , ϵ -diamino-derivative of caproic acid, which itself is the next fatty acid above valeric acid :—



Proteins are known which contain no lysine, and if these be fed to young rats the animals appear to remain quite healthy, but fail to grow up; they stay in a glorious condition of lasting youth, until growth is recommenced by the feeding of a normal full amino-acid diet. The amino-acids of the group to which lysine belongs seem not to be essential for the maintenance of the living

structure when once it is fully formed, but they are necessary for building it up from the beginning. The adult body must evidently possess a faculty that is lacking in the young—the faculty, that is, of synthesising lysine from other amino-acids. This could hardly be illustrated more strikingly than by the observation that a lactating female rat receiving a diet deficient in lysine produces by some kind of chemical dexterity milk that is complete with regard to its amino-acids, for on it her young grow normally until they are weaned. If they then receive the deficient diet their growth is arrested, for they do not possess the skill in chemical synthesis that enabled their mother to rear them.

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In addition to CATHCART's monograph on Protein Metabolism, to which we have already referred (p. 50), more recent works dealing with this subject are:

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T. B. OSBORNE and L. B. MENDEL's articles on the importance of various amino-acids for growth are to be found in the *Journal of Biological Chemistry* from vol. 12 (1912) on, and are summarised in a popular form in

MENDEL, L. B.: *Nutrition: the Chemistry of Life*. (Oxford and Yale Univ. Presses.)

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DODDS, E. C., and DICKENS, F.: *The Chemical and Physiological Properties of the Internal Secretions*. (Oxford Univ. Press.)

A student of organic chemistry who is interested in the synthesis of substances occurring in living organisms will delight in HARINGTON's accounts of his recent synthesis of thyroxine:

Biochemical Journal, vol. 20 (1926), pp. 293 and 300, and vol. 21 (1927), p. 169;

HARINGTON, C. R.: *The Thyroid Gland: its Chemistry and Physiology*. (Oxford Univ. Press.)

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CHAPTER VII

THE BIOCHEMISTRY OF THE PURINES : NUCLEOPROTEINS : URIC ACID

"This racks the joints, this fires the veins."—*Gray*.

THERE remains yet a special group of proteins whose chemistry and metabolism we must consider before we shall have completed our study of nitrogenous substances. We refer to the **nucleoproteins** which are characteristic constituents of the nuclei of cells. They are extracted from tissues containing abundance of nuclei or nuclear matter (thymus, yeast) by means of dilute alkaline solutions, or even water, the alkaline salts of the tissue itself serving in the latter case to carry the nucleoproteins into solution, from whence they are afterwards precipitated by making the extract faintly acid (cf. the account of the solubility relationships of the proteins given in Chapter II). As we there mentioned, the nucleoproteins are examples of **conjugated proteins**—substances whose complex molecules contain not only protein, but also non-protein groups. Without giving details at the moment, we will mention simply that the non-protein group of the nucleoprotein molecule contains, among other constituents, substances known as the **purine bases**. The importance of these bases from the physiological point of view is that they are closely related chemically to **uric acid**, and that during the course of metabolism they become converted into this substance. We may say, then, that just as urea and creatinine are characteristic products of the breakdown of ordinary proteins, so **uric acid** is the characteristic end-product of the metabolism of nucleoproteins. Knowing this, the

reader who has followed the argument contained in the previous chapters will have no difficulty in appreciating the significance and importance of a study of the formation and output of uric acid from the body. For, apart from secondary factors, the amount of uric acid in the urine is a measure of the total amount of nucleoprotein suffering decomposition.

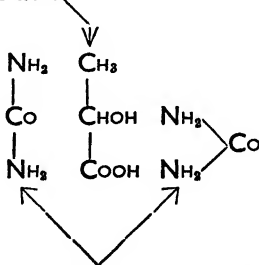
It will be most convenient if we consider the chemistry of this end-product uric acid first. It is a white crystalline solid that is only very sparingly soluble in aqueous fluids. It is not surprising, on this account, that it should have attracted attention as a constituent of urinary calculi some years before the much more abundant but much more soluble urea was recognised as a constituent of urine. In consequence of its insolubility uric acid readily separates from the blood into the joints and tissues, so giving rise to arthritis and gout, and thus furnishing a valuable source of income to the medical profession. Uric acid is precipitated quantitatively as a gelatinous ammonium urate by first making its solution alkaline with ammonia and then saturating it with ammonium chloride—a property made use of in preparing specimens of uric acid from urine, the free uric acid being easily liberated from the ammonium urate by the action of hydrochloric acid. This method of precipitation was also made use of in an older process for the estimation of uric acid before the modern colorimetric method, directly applicable to urine, was devised. By virtue of the fact that uric acid is a fairly powerful reducing agent the acid obtained by precipitation was estimated by titration with permanganate. The reducing properties of uric acid are further shown by the facts that it liberates metallic silver from silver salts (Schiff's test), and reduces Fehling's

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solution (p. 151)—a very important point to bear in mind when one is testing a urine or other fluid for sugar. The most characteristic qualitative reaction given by uric acid is, however, that known as the **murexide** test: this consists in moistening a little of the material under investigation with strong nitric acid and evaporating to dryness. If uric acid was originally present a red solid residue is obtained, and this dissolves in a drop or two of ammonia, giving a striking purple solution that resembles in colour, but not in chemical constitution, the Tyrian purple that the ancients used to obtain from the sea-snail *Murex*—hence the name of the test.

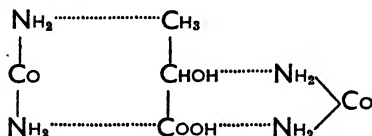
Doubtless the reader will have already noticed in his reading the complicated ring structure of the formula of uric acid, and may have been struck by the apparent difficulty of remembering the arrangement of the atoms. But the difficulty vanishes if we remember that the chemist knows several methods by which he can synthesise uric acid in the laboratory, and that of these the easiest to understand is that in which he starts from two molecules of urea and a molecule of some acid containing a three-carbon chain—we will, in point of fact, take lactic acid as our example.

Here is the lactic acid



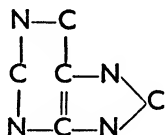
and here the two molecules of urea. Unimportant atoms

are in small type. It will perhaps be objected that we have arranged these molecules in such a way as to suit our immediate purpose. Be that as it may—the point is that by appropriate means the chemist can induce these molecules to react in a way that can be represented like this :—

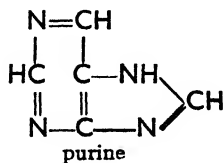


and so to give rise to a product containing the same double ring composed of nitrogen and carbon atoms which is characteristic of the molecule of uric acid, and from which uric acid itself can be readily obtained.

In this way it is easy to remember that the fundamental atoms in the molecule are arranged in a double ring structure thus :—

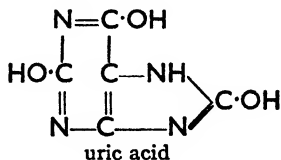


which is known as the **purine ring**. If we add hydrogen atoms to complete the valencies we obtain the formula for **purine** itself—a basic substance which contains only carbon, hydrogen and nitrogen, and is the mother-substance of all the purine derivatives we are considering.

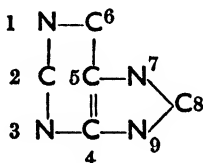


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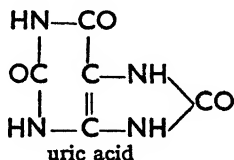
Now uric acid is a trihydroxy-derivative of purine in which the hydroxyl groups are all attached to carbon atoms thus :—



As a matter of convenience, numbers are usually assigned to the atoms in the purine ring, and by convention it is agreed to label them in the following order :—



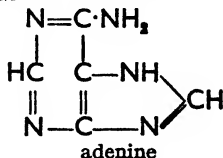
This numbering is purely arbitrary, but it is, of course, necessary to be able to refer to the positions of substituting groups in the molecule. Using this nomenclature we should say that uric acid is 2:6:8-trihydroxy-purine. As a matter of fact, one often sees its formula written in a slightly different way which expresses better its relation to two molecules of urea and a three-carbon chain :—



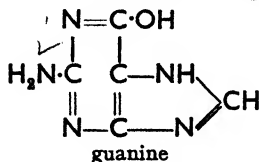
but this does not affect our present argument. Chemists believe that in this case the hydrogen atoms of the hydroxyl groups are capable of wandering from their

appropriate oxygen atoms to the adjacent nitrogen atoms under some circumstances, so that it depends upon the conditions which formula the uric acid will obey. This behaviour is displayed by hydrogen atoms in some other substances ; it is called tautomerism.

The other important purine derivatives with which we have to deal are the purine bases. These are amino-derivatives of the purine ring. Among them the most important are **adenine**, which is 6-aminopurine :—



and **guanine**, which contains a hydroxyl group as well as an amino-group and is 2-amino-6-hydroxypurine :—



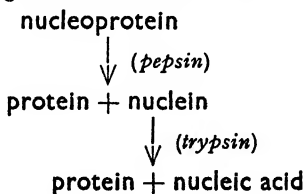
It should be noted that the $\cdot\text{NH}_2$ and $\cdot\text{OH}$ groups in these substances are attached to carbon atoms which carry $\cdot\text{OH}$ groups in the uric acid molecule.

Adenine and guanine are the purine bases that occur most commonly in the non-protein groups of nucleoproteins; it is most important to realise that it is from these substances that the uric acid of the urine is derived by a series of metabolic changes which we shall presently study. Incidentally, as a matter of interest, one might mention that the drug **caffein** is also a purine derivative ; it is a trimethyl derivative of xanthine—a substance we

shall meet later. Not that caffeine enters into purine metabolism to any great extent, but as it occurs in such beverages as tea and coffee, and, furthermore, is a drug that finds use in physiological experiment and in medicine, it is worthy of a passing notice.

Having dealt with the relationships of the chief purine substances that concern us, we are now equipped with the fundamental information we need in order to understand the constitution of the nucleoproteins. Seeing that these form the essential constituents of nuclear matter, and that the nucleus is in many ways the most complicated part of the cell, it is not surprising to find that nucleoproteins are among the most complicated substances with which we have to deal.

As we have already mentioned, a nucleoprotein is what we term a conjugated protein—that is, that its molecule consists of a protein molecule combined with a group which is not a protein. In the particular case of the nucleoproteins we have two protein molecules joined to a non-protein substance called **nucleic acid**. This is proved by submitting nucleoproteins to the action of the digestive proteolytic enzymes of the alimentary canal in turn. When pepsin acts on a nucleoprotein a certain amount of protein is digested away and an insoluble residue called nuclein remains. This still contains protein which can be completely removed by the action of trypsin, leaving the free nucleic acid thus :—



It is quite easy to remember these changes as they are brought about by the protein-splitting enzymes of the alimentary canal acting in the order in which the nucleoproteins of the food would ordinarily meet them during the course of digestion. In order to prepare nucleic acid it is, however, usually more convenient to bring about these changes in one step by heating the nucleoproteins with fairly strong caustic soda solution. Nucleic acid itself is a complicated substance. When it is boiled with dilute mineral acids it breaks up into phosphoric acid, sugars and the purine bases, but more gentle treatment reveals the fact that these constituents are united into groups termed **nucleotides**, each consisting of a single molecule each of phosphoric acid, sugar and purine base, thus :—

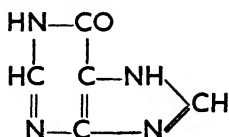
phosphoric acid—sugar—purine base.

It is to be noted that in each nucleotide the sugar molecule occupies as it were the central position, being united on the one hand to the phosphoric acid (probably by an ester linkage—we shall meet again such compounds of sugar and phosphoric acid, for example, hexose phosphate, p. 192), and on the other to the purine base by a linkage that must involve the active reducing group of the sugar inasmuch as nucleic acids are devoid of reducing properties.

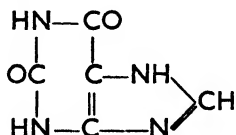
Different nucleotides may contain different sugars and purine (or even other) bases, so that there is a considerable range of structure among these constituent units of the nucleic acid molecule. It is found, however, that the nucleic acid itself obtained from no matter what plant source is always composed of one and the same set of nucleotides, while that from all animal sources is composed

of a similarly constant but different set. We thus have two but only two different nucleic acids—plant nucleic acid and animal nucleic acid, or, as they are usually called, after their respective usual sources, yeast nucleic acid and thymus nucleic acid. But there are many different nucleoproteins, for there are, of course, enormous possibilities of variation of structure among the protein groups with which one or other of these nucleic acids may be combined.

As we have seen, pepsin and trypsin leave the nucleic acids unattacked, but when these arrive in the small intestine they are broken down by an array of enzymes present in the intestinal juice first into their constituent nucleotides and then, by further decomposition of these nucleotides into phosphoric acid, which goes to form the inorganic phosphates of the urine, sugars which, when carried to the liver, may form glycogen, and purine bases, in which we are further interested. The purine bases are absorbed into the blood stream, and are carried to the liver and other tissues. The student who is familiar with the metabolism of proteins will feel no surprise at learning that the next process to which these amino-derivatives are subjected is one of deamination: the decomposition of the purine bases is begun by the splitting off of the amino-group. Now in the case of the amino-acids we found keto-acids and ammonia to be formed as a result of deamination. Exactly in the same kind of way keto-derivatives are produced in the tissues by the deamination of adenine and guanine. In the substances so formed the original position of the amino-group is now marked by the :CO group in the ring. Thus, the substance formed from adenine has the formula



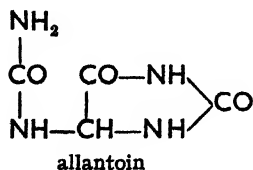
and is called **hypoxanthine**, because it is lower in degree of oxidation than that which, being formed from guanine by a similar change, has the formula



and is called **xanthine** on account of the yellow residue it gives on evaporation with nitric acid (Gr. *ξανθός* = yellow). (It will be remembered that guanine starts with one atom of oxygen already in its molecule.) The conversion of xanthine (a dioxy-derivative) and hypoxanthine (a monooxy-derivative) into the trioxy-derivative uric acid requires a process of oxidation, which in man seems to take place in the liver only. Now while these changes have been suffered by the food proteins, the tissue nucleoproteins have also been breaking down to some extent. Nucleic acid has been liberated from them and split up into nucleotides, and these further into phosphoric acid, sugars and purine bases. These purine bases, which are, of course, endogenous, become deaminated, and the resulting xanthine and hypoxanthine are oxidised, so that finally we have not only the uric acid that has been formed from food nucleoproteins, but also some that has been formed by the breakdown of the nucleoproteins of the tissues. In other words, part of the uric acid excreted is of exogenous and part of endogenous origin.

The reader will have observed that we have not yet said anything very definite about the particular organs in which all these changes occur. The reason for our vagueness is that the site at which each particular reaction occurs has been found to vary from animal to animal. It would take us into too great detail to mention all the facts that have been established with regard to the distribution of the enzymes concerned with the metabolism of purine derivatives, but a few striking cases may be of interest. It is found, for example, that man can metabolise free adenine only with difficulty so that this purine base occurs in small quantities in normal urine. On the other hand the pig seems to be deficient in the power of metabolising guanine so that this substance is liable to crystallise out in the tissues of this animal giving it a kind of "guanine gout." The ox, however, possesses in its liver all the enzymes necessary for all the stages in purine metabolism.

To resume the main line of our argument—the total uric acid formed in the body is usually not excreted as such in the urine. In most mammals the liver contains yet another enzyme in addition to those already mentioned, and this brings about the further oxidation of uric acid to a substance called **allantoin**, whose molecule contains the remains of a purine ring which has been, so to speak, burnt into and opened :—



The name allantoin is derived from that of the sausage-shaped embryonic bladder or allantois in whose contents the substance was first discovered (Gr. ἀλλᾶς, ἀλλάντος = sausage). This allantoin has the great advantage over uric acid as an excretory product that it is much more soluble. To dissolve one gram of allantoin only 160 c.c. of water are needed as opposed to the 40,000 c.c. that are required to dissolve a gram of uric acid. In ourselves, however, in our closer simian relatives and, curiously enough, in the Dalmatian coach-hound (the "plum pudding" dog) this uric-acid-destroying enzyme or **uricase**, is lacking, so that we have to excrete in the urine all the uric acid we produce during the metabolism of our nucleoproteins. On an average diet, however, it amounts only to about 0.75 grm. per day.

In contrast with this, uric acid is the main nitrogenous constituent of the semi-solid urine of birds and reptiles. It was formerly supposed that in these animals the uric acid was synthesised from urea and lactic acid as shown on p. 108. But now it is found that the rate of uric acid production by slices of hen's liver is not influenced by the addition of urea, although it is accelerated by both ammonium salts and lactic acid; from this it appears that ammonium lactate is the precursor of the uric acid that the bird excretes.

There is no doubt that man and other mammals can also synthesise the purine ring—in this case from amino-acids. The classical example is that of a "healthy young woman" who when kept for fifty days on a diet poor in purines not only excreted 15 grm. more of uric acid than could be accounted for by the food, but actually put on 4 kilos. in weight. She had evidently synthesised a considerable number of purine rings during the experiment!

The conclusion is, then, that the mammal has the power of synthesising the purine ring from some substances which themselves do not contain it ready formed. Some evidence as to the raw materials used in this synthesis has been obtained by an experiment in which a number of rats were fed on food containing no arginine and no histidine.

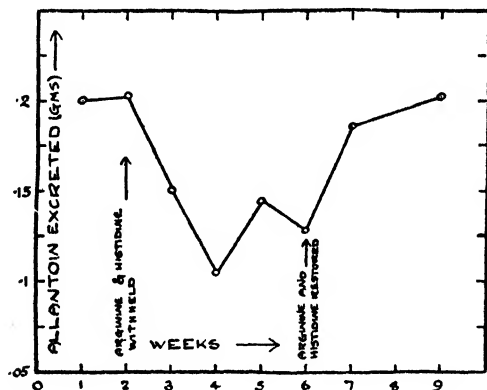
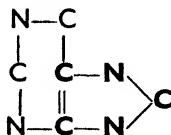


FIG. 7. The reduction of the output of the purine excretory product allantoin on withholding both arginine and histidine from the diet of a rat.

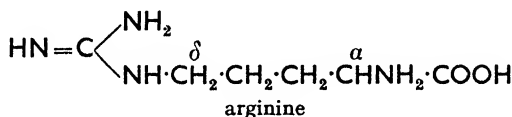
The allantoin excretion returns to normal when these amino-acids are once more added to the food. (Plotted from the results of an experiment by Hopkins.)

It was found then that the output of purines in the urine of the rats was very much reduced during the experimental period, but returned again to normal when the full diet containing histidine and arginine was resumed (see Fig. 7). The omission of other amino-acids from the diet produced no such effect. It is not difficult to see how histidine and arginine might possibly be the precursors of the purine ring in the body. Histidine, it will be remembered,

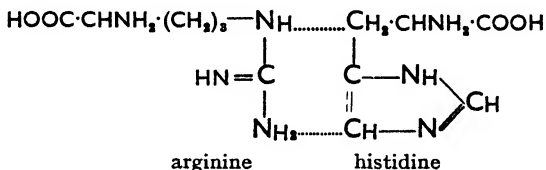
contains an iminazole ring, and in one part of the purine ring we have also that combination of carbon and nitrogen atoms which constitutes an iminazole ring, thus :—



Arginine, it will be remembered from p. 65, is α -amino- δ -guanidine-valeric acid :—



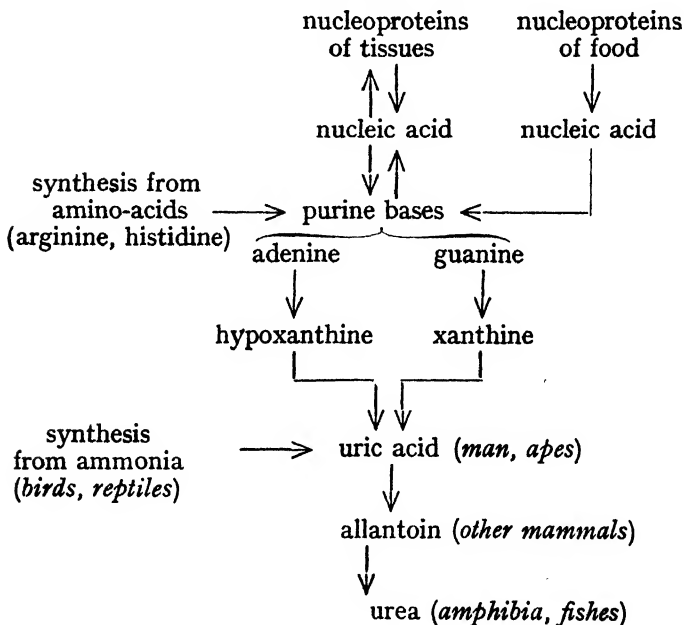
By re-writing the formulæ it is easy to see how arginine and histidine might conceivably be useful in building up the purine ring, thus :—



It might be pointed out also that the same synthesis of the purine ring from non-purine substances can be observed during the incubation of an egg. For as the nuclei divide more and more nucleoproteins are built up, so that the fully developed chick contains a much greater weight of purine derivatives than the original egg, although no food has been supplied from outside.

SUMMARY

The following scheme may assist the reader to summarise our account of the chemical changes undergone by the chief purine derivatives in the body.



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and

JONES, W.: *Nucleic Acids*. (Monographs on Biochemistry; London: Longmans, Green & Co.)

Also in the same series is a volume by

DAKIN, H. D.: *Oxidations and Reductions in the Animal Body*,

which contains a chapter on the production of uric acid. This is extended in a paper by the same author:

"*Physiological Oxidations*" (*Physiological Reviews*, vol. 1 (1921), p. 394).

A survey of the subject of purine metabolism is given by

ROSE, W. C., in "*Purine Metabolism*" (*Physiological Reviews*, vol. 3 (1923), p. 544),

and later work is dealt with by

KREBS, H. A.: "*Urea formation in the animal Body*" (*Ergebn. d. Enzymforschung*, vol. 3 (1934), p. 247).

The synthesis of purine derivatives from amino-acids is dealt with by ACKROYD and HOPKINS in an article entitled:

"*Feeding Experiments with Deficiencies in the Amino-Acid Supply. Arginine and Histidine as Possible Precursors of Purines*" (*Biochemical Journal*, vol. 10 (1916), p. 551).

For those interested in the relation between biochemistry and genetics:

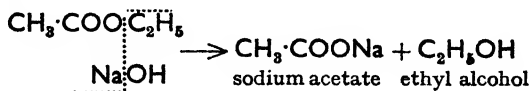
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CHAPTER VIII

FATS AND THEIR METABOLISM: PHOSPHOLIPIDES

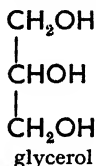
"We fat all creatures else to fat us, and we fat ourselves for maggots."—*Hamlet*.

As usual in dealing with the biochemistry of any group of substances we will begin with a brief outline of the pure chemistry of the materials in question. From his previous reading of organic chemistry the student will remember that just as we can take an acid—acetic $\text{CH}_3\cdot\text{COOH}$, for example—and neutralise it with caustic soda and obtain its sodium salt, $\text{CH}_3\cdot\text{COONa}$, so in somewhat similar manner we can cause the acid to react with an alcohol and obtain a "salt" of an organic radicle, the alcohol playing the part of weak base. If we use ethyl alcohol, then acetic acid will give us ethyl acetate— $\text{CH}_3\cdot\text{COOC}_2\text{H}_5$. Such a salt of an organic radicle is called an ester. One of the most characteristic reactions shown by an ester is that which occurs when it is boiled up with caustic soda; the ester is thereby hydrolysed, its alcohol being liberated and its acid remaining as the sodium salt. Still taking ethyl acetate as our example, we can represent the process thus :—

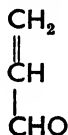


Now the fats are simply esters : they are salts formed by a union of an alcohol with acids.

The alcohol that occurs in the fats is not the simple ethyl alcohol but the somewhat more complicated **glycerol** containing three $\cdot\text{OH}$ groups :—

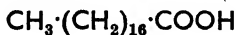


This is a colourless, viscid, sweet-tasting, water-miscible liquid which has been known to crystallise by prolonged cooling. It is known *unsystematically* as "glycerine." (In the systematic nomenclature of organic chemistry the names of hydroxy derivatives end in "ol," the ending "ine" being reserved for basic nitrogenous substances such as amines.) In analysis glycerol is easily identified by the fact that on heating with dehydrating agents such as potassium hydrogen sulphate it loses two molecules of water and forms the aldehyde **acrolein** which has a very characteristic acrid choking smell (L. *acris*=sharp).



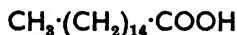
The commonest acids whose glycerol esters compose ordinary fats are :—

(1) **Stearic acid** (Gr. *στέαρ*=tallow, suet), which is a typical member of the fatty series, with a long straight chain composed of eighteen carbon atoms, that may for brevity be written thus :—



This is a waxy white solid that can be crystallised from organic solvents ; it is the chief constituent of so-called "stearine" candles.

(2) **Palmitic acid**, which is the next member *but one* below stearic acid, and so contains a carbon chain *two* atoms shorter :—



It occurs abundantly in palm oil and resembles stearic acid in its general properties.

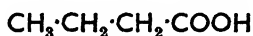
(3) **Oleic acid**, which is an oily liquid. Its molecule contains the same number of carbon atoms as stearic acid, but two hydrogen atoms less. It is therefore unsaturated, and since ozone splits the molecule into two portions each with nine carbon atoms the double bond presumably occurs in the middle of the chain :—



As acids these substances are feeble, but they dissolve fairly readily, forming salts, in aqueous solutions of caustic alkalis, and so can be distinguished from their parent fats.

Seeing that the glycerol molecule contains three hydroxyl groups, it is evident that it will combine with three molecules of either of these acids. The ester formed from glycerol and three molecules of stearic acid is called **tri-stearin**; that containing three molecules of palmitic acid — **tri-palmitin**; and, lastly, that containing three molecules of oleic acid is referred to as **tri-olein**. The naturally-occurring fatty materials are usually mixtures in various proportions of these three simple glycerides, but they often contain mixed glycerides — with different fatty acids in the same molecule — as well. In general it may be said that fats containing abundance of the esters of stearic and palmitic acids have higher melting-points than those containing much oleic acid,

so that the greater the proportion of oleic acid in a fat the softer it will be. Thus olive oil which is very rich in oleic acid is a liquid fat, pigs' fat (lard) which contains less of oleic acid is semi-fluid, while mutton fat (suet) which contains least oleic acid of all is a firm solid at ordinary temperatures. In butter, which is, of course, simply milk fat, there occur glycerides of lower acids such as the simple four carbon **butyric acid** :—



Under normal circumstances the stored fat of each animal is a definite mixture of glycerides, characteristic of its species, but when excessive quantities of foreign fats are administered these tend to be stored unchanged, so that the normal composition of the fat is departed from.

We have mentioned that if we take an ester and stew it up with caustic soda we get a sodium salt and the free alcohol. This is true of the esters that make up fats : if these are boiled for some time with caustic soda, the alcohol glycerol is liberated and the sodium salts of the fatty acids present are formed. This process is called saponification—soap formation—because it is the process fundamental to the making of soap. Ordinary soap consists of the sodium salts of stearic and palmitic acids, and it is got by boiling up fats with caustic soda. Incidentally, of course, glycerol is liberated at the same time, and that is why “glycerine” is always produced at the soap works. The sodium soaps of these fatty acids are all readily soluble in water, giving alkaline solutions whose valuable detergent properties are well known. When they are treated with a mineral acid the fatty acid is liberated and floats up to the surface of the solution—it would be a contradiction in terms to say that it is

"precipitated." The calcium and magnesium soaps of these higher fatty acids are insoluble and separate out as the scum that is formed when soap is used with hard water. Their green insoluble copper salts are formed when soap is treated with a copper nitrate solution, and this reaction is used in clinical investigations as a microscopic test for distinguishing between soaps and undigested fat or free fatty acids in faeces.

The fats themselves are usually detected and estimated by taking advantage of their physical properties. They can be extracted from dried foods or other materials by boiling with strong alcohol, in which case they can be precipitated as an emulsion by pouring the alcoholic solution into water; or they may be extracted by boiling with ether, in which case the fat is recovered unchanged by evaporation of the solvent. That it is fat is shown by its power of making a "grease spot" on a piece of paper. The only other common substance that will make paper translucent in a somewhat similar way is glycerol, but this is not soluble in ether, and so would not be extracted, and in any case it can be easily washed out of the paper by water. Furthermore a stain due to a fat spot can be stained with fat-soluble dyes such as Sudan III just as can the fat in a histological preparation. Fat is estimated by making the ether extraction quantitative usually in some form of continuous extractor such as the Soxhlet apparatus, and then evaporating the ethereal extract in a tared flask.

The identification of a particular fat is a more difficult matter. Complete hydrolysis followed by separation and estimation of all the liberated fatty acids is too complicated a process for routine investigations. However, by carrying out the process of saponification quantitatively,

using a known amount of caustic alkali and titrating back that which remains in excess, we obtain a measure of the amount of alkali required for the neutralisation of the total fatty acids liberated from a known amount of a fat. It is evident that this "saponification value" will differ according to the particular proportions in which the various fatty acids are present, and so may be used for identifying a naturally-occurring fat. Another method of identifying a fat depends upon the circumstance, so familiar to students of organic chemistry, that one of the most typical points in the behaviour of unsaturated compounds is that they combine directly with halogens—iodine, for example. In particular, oleic acid and its esters exhibit this property, so that the amount of iodine with which a fat will combine is a measure of the percentage of oleic or similar unsaturated acids present in it. Hence the value of determining the "iodine number" in the investigation of fats. Yet a third method depends on the estimation of the percentage of volatile fatty acids—butyric and others—as contrasted with the higher non-volatile ones. This is useful for distinguishing between butter which contains, and margarine, which does not contain, such volatile acids. (Margarine is obtained by the "hardening," i.e. reduction with hydrogen in the presence of nickel as catalyst, of cheap vegetable oils; the unsaturated liquid glycerides being thereby converted to solid saturated fats.)

Let us now consider the sources whence the body obtains fats. The most direct source of supply is the pre-formed fat in the food. Now although the body utilises the fat of the food as fat, and does not require to convert it into any other substance before it can store it or use it, yet the fat of the food is submitted to a very

definite process of digestion before it is absorbed. This digestive process consists in the hydrolysis of the fat into glycerol and fatty acids under the influence of fat-splitting enzymes, or **lipases**. A certain amount of such an enzyme is secreted by the stomach, but its action is probably slight on account of the lack of emulsification of the fat in the acid medium. The chief lipase that occurs in the alimentary canal is that secreted in the pancreatic juice; it long ago received the name of **steapsin**. This exerts a very powerful hydrolytic action on fats, and the conditions of its action are favoured in a very marked degree by the properties of the bile which is poured into the duodenum simultaneously with the pancreatic juice. In the first place the bile is strongly alkaline, so that the pancreatic lipase acts in an alkaline medium. This means that the fatty acids, as soon as they are liberated, are converted into soaps, which, in dissolving, set up diffusion currents that break up the fat particles into the fine globules of an emulsion, and so expose a very large total surface of fat to the action of the enzyme. This effect is very easily demonstrated by floating a drop of olive oil containing a small quantity of oleic acid on to the surface of sodium carbonate solution: the oil is seen to pass spontaneously into a milky emulsion. Then again, both the soaps and the bile have the property of stabilising such an emulsion—that is, of preventing the reunion of the separate globules. This they do by lowering the surface tension at the fat/water interface—by diminishing, that is, the force that causes the surfaces of liquids to shrink to the smallest possible area and so causes the droplets of an emulsion to coalesce and form larger drops. Any substance that lowers surface tension tends to accumulate in the surface

(see p. 378), so that in this case the fat globules become surrounded with a layer of soap that prevents their coalescence with their neighbours. This property of bile can be easily illustrated by trying to write on oiled paper. Ordinary ink simply balls itself up into separate droplets on the greasy surface, but if a little bile is added to it it will spread quite easily, and the writing can be carried out. Exactly the same property is utilised in testing for bile in a patient's urine: flowers of sulphur are sprinkled on to the surface of the liquid; if the urine is normal they float unwetted; if it contains bile they promptly sink to the bottom. Bile possesses these remarkable properties by virtue of the **bile salts** it contains. These are the sodium salts of **glycocholic** and **taurocholic acids** — complex "bile acids" whose essential component **cholic acid** is related in structure to cholesterol which also occurs in bile (p. 147). A further use of these bile salts is that they keep in solution the otherwise insoluble fatty acids, so that these are not precipitated even in the more distal portions of the small intestine where the reaction of the food mass is no longer so alkaline as in the duodenum. It remains to mention that the digestion of fat is continued in these portions of the intestine by means of a further lipase present in the intestinal juice.

We must now consider the mechanism by means of which these products of the hydrolysis of fats are absorbed from the alimentary canal. It will be remembered that the amino-acids resulting from the digestion of proteins are absorbed into the blood stream, through the capillary blood vessels of the villi of the small intestine; but the fats are absorbed by a different route. The mixture of glycerol and fatty acids passes into the columnar cells lining

the villi, and there, in these cells, we find that the fatty acids and the glycerol that have been absorbed together are re-combined to form fat, which accumulates in the form of droplets. It thus appears that the chief object, if we may so express it, in hydrolysing fat in the alimentary canal to fatty acid and glycerol, is to produce substances sufficiently soluble to pass through the limiting membrane of the intestinal cell. If the cells of the intestine are examined histologically during the absorption of fat, it is found that as the fat globules pass from the edge of the cell that was facing the intestinal cavity they become larger and larger. Escaping from the opposite end of the cell, they find their way across to the central cavity of the villus which, on account of the presence of fat in the fluid it contains, is usually referred to as a central lacteal. This vessel is really a lymph channel, and its contained fluid—known as chyle—having become loaded up with fat globules, passes round in the lymphatic system, and finally enters the blood by the thoracic duct. If the fat is not to be used at once for the purpose of obtaining energy it is stored unchanged in the fat depôts which are chiefly in the mesentery and the subcutaneous connective tissues. The absorption of digested fat is, however, even under normal conditions far from complete, for the fæces contain about a quarter of their dry weight of ether-soluble material, most of which is made up of fatty acids together with some undigested fat, and in cases of obstruction of the bile duct the proportion is still greater, showing that bile plays an important part in the absorption as well as the digestion of fat.

Now we have already mentioned that the fat occurring in the fat depôts of an animal is, under normal

circumstances, a definite mixture of stearin, palmitin and olein in fixed proportions, which are different for the various species of animals. It therefore follows that if an animal of one species is fed on the fat of a different species then there must occur a transformation of the mixture of esters present in the food fat into that different mixture of esters which constitutes the *depôt* fat characteristic of the animal that has received the food. This transformation occurs even in the cells of the villi in which the fat is resynthesised during absorption, for the fat in the chyle is found to agree in composition with the *depôt* fat rather than with the food fat. But the power which the body possesses of bringing about this change is limited, for if a large excess of a foreign fat be fed, some of this may be deposited unchanged in composition. Thus it is possible to replace the normal fat of a dog's body by a much softer fat of very much lower melting-point by feeding it with large quantities of linseed oil, or to bring about the storage of a harder fat than normal by including large quantities of mutton fat in the diet. In a similar way a foreign fat fed to a lactating animal may appear in its milk. This mixture of esters characteristic of a species may arise not only from the fat, but also from other constituents of the food—more particularly from the carbohydrates. The fattening of cattle on a diet containing abundance of carbohydrate is a matter of common experience ; it has been a subject of exact enquiry in the case of the pig. Two pigs of the same litter, and of as nearly as possible equal weights, were taken ; one was slaughtered at once, and the amount of fat in its body was estimated. It is assumed that the other similar pig started off with this same amount of fat. This second pig was then brought up on a carefully analysed diet for some

months. At the end of this time it was found that there was much more fat in its body than was present at the start, and that the increase was much more than could be accounted for by the amount of fat it had taken in with its food; therefore the carbohydrates of the diet must have given rise to fat.

It is now generally accepted that such a synthesis of fat from carbohydrate takes place very extensively in the bodies of all animals, including man, but in spite of this it is a matter of experience that humans, at all events, crave a certain minimum of pre-formed fat in their diets—possibly because the mixture of fatty acids synthesised from carbohydrates does not include certain acids with branched, and in other ways special, chains that occur in the natural fats.

The question as to the extent to which proteins give rise to fat in the body is a much debated one. At first they were thought to do so because an animal on a lean meat diet most certainly puts on fat. But then it was realised that even in lean meat the muscle fibres contain a sufficiently large amount of fat to account for all that is put on, so that the proteins of the food have not necessarily given rise to new fat. Then again it is found that the fat that appears during the so-called fatty degeneration of organs—of the liver, for example, as a result of phosphorus poisoning—is either transported from the fat depôts or else is liberated from some masked but pre-existing form in the organs themselves, and so, again, is not manufactured from proteins of the tissues. The only creatures that seem definitely to possess the power of converting proteins into fats are maggots. This is a rather surprising conclusion, for we know that proteins readily give rise to carbohydrates in the body

(formation of glycogen from amino-acids, p. 73), and it might be thought that these carbohydrates might, like carbohydrates taken in with the food, be further converted into fat. But apparently in the mammalian body, at least, it is not so, and excess of protein food gives rise to glycogen, but not fat.

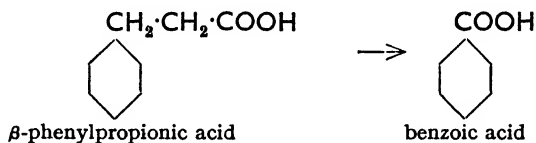
Mention above of the masked fat in tissues, i.e. fat not demonstrable by histological staining with osmic acid, but, nevertheless, extractable from the tissues by alcohol, reminds us that although the most obvious use of fat is as fuel, it is also an essential constituent of living matter. Not all the fat of the body is mobilised when the need for combustible material arises; in spite of starvation the tissues retain a constant element of fat that thus appears to be essential for their life. This is illustrated in an extreme degree by the brain, which during starvation does not give up any appreciable amount of its relatively abundant fat content for the general purposes of the body.

Nevertheless, the essential chemical function of most of the fat in the body is to act as fuel to the tissues. In this connection it should be pointed out that fat is a very economical form in which to store fuel. For the molecule of a fat consists almost entirely of carbon and hydrogen atoms. It possesses but few oxygen atoms—far less relatively than are present in a carbohydrate molecule—and no incombustible nitrogen such as is present in a protein molecule. Thus it comes about that, weight for weight, fat contains much more potential chemical energy than either carbohydrate or protein. A gram of fat on oxidation gives off 9.5 large Calories, as compared with 5.6 Calories obtainable from a gram of protein, and only 4.2 Calories from a gram of carbohydrate.

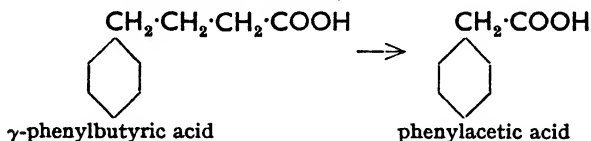
The attempt to elucidate the stages through which a long

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fatty acid chain such as exists in stearic acid is finally oxidised in the body to carbon dioxide and water provides us with one of the most interesting of biochemical problems. Our definite knowledge on the subject dates from 1904, when Knoop hit upon the idea of "fixing" one of the carbon atoms of a fatty acid chain by attaching to it a benzene ring and so rendering it immune to the oxidative processes of the tissues. He found that if he administered to an animal β -phenylpropionic acid two carbon atoms of the side chain were removed and benzoic acid was produced and excreted (in combination with glycine) in the urine.

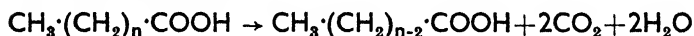


If, on the other hand, he fed the ^{animal} acid with one more carbon atom in the side chain, namely, γ -phenylbutyric acid, the excreted product was found to be the glycine derivative of phenylacetic acid:—



The compound with still another carbon atom in the side chain gave, however, benzoic acid as its oxidation product, and that with yet one more carbon atom gave phenylacetic acid again. And so on—a side chain containing an odd number of carbon atoms gave always benzoic acid, and a side chain with an even number of

carbon atoms gave phenylacetic acid. This striking regularity shows that in these compounds the carbon atoms can be removed by oxidation in the body only *two* at a time: if they could be removed singly there would be no reason why phenylacetic acid should not lose one more carbon atom from its side chain so that benzoic acid would be the end-product from all, and not merely alternate, phenyl-fatty acids. It is supposed from this that the body deals with the long chains of fatty acids by a similar process of β -oxidation—that is, that it, as it were, burns off the carbon atoms two at a time, leaving the carbon atom that was in the β -position to the original carboxyl group as the centre of the carboxyl group of a fatty acid containing a chain of *two* less carbon atoms—and so on until the whole chain is completely oxidised to carbon dioxide and water. The process may be represented thus:—

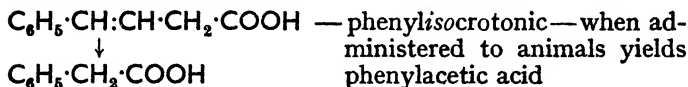


More recently, however, it has been found that if the phenyl-derivatives of 9- or 10-carbon fatty acids—of substances, that is, that resemble more closely the actual fatty acids that the body has to deal with than do the relatively simple substances investigated by Knoop—are administered, the yields of benzoic and phenylacetic acids respectively are much less than from the shorter chained compounds. This suggests that the longer fatty acid chains may be dealt with by other processes in addition to the simple β -oxidation. Nevertheless, by the use of a suitable oxidising agent such as hydrogen peroxide, it can be shown that even outside the body even the long-chained fatty acids such as stearic have a tendency to oxidation in the β -position, the main,

though not the sole, products being in this case the corresponding β -keto-acids.

Of course the removal (ultimately as carbon dioxide and water) of the last two groups of a fatty acid molecule is a process that must involve several stages, and the question naturally arises as to the details of its course. It might be, for example, that the first step consists in the introduction of an $\cdot\text{OH}$ group in the β -position, although this is not very likely as hydroxy-acids in general are not very easily oxidised in the body. Or, more probably, the β -group might be converted into a keto group; or, lastly, by oxidative removal of hydrogen a double bond might be established between the α - and β -carbon atoms. The difficulty in deciding between these possibilities arises from the fact that when such an acid as phenylpropionic is administered to an animal small quantities of the corresponding hydroxy-, keto- and unsaturated acids are all found, in addition to benzoic acid, in its urine. With regard to the formation of the unsaturated acids it has been pointed out that during such conditions as starvation and pregnancy, in which there are considerable demands on the body's store of fat, the liver becomes loaded with fat that differs from the ordinary *dépôt* fat by containing a much greater proportion of unsaturated acids. This has been interpreted by supposing that the first step in the oxidation of a fatty acid chain is the introduction of unsaturated linkages into the molecule. But it by no means follows that unsaturated compounds are distinguished in the body by that easy oxidisability at the double bond that they show *in vitro*, and cases are known in which it seems as if the first step in the utilisation of an unsaturated substance in the body consists in its

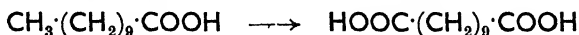
reduction to the corresponding saturated compound. This can be shown by choosing a substance in which the unsaturated linkage is in such a position that β -oxidation will yield a product different from that resulting from rupture at the double bond. For example, the acid



(just as if it were first reduced to phenylbutyric acid and then oxidised in the β -position) and not benzoic acid, as would be expected if the chain had been ruptured at the double bond. This leaves the β -keto-acid as the most probable direct intermediate compound formed during β -oxidation—a view for which we shall meet with further evidence when we come to consider the derangement of fat metabolism that occurs during the condition of diabetes. Even, however, if we were quite certain as to the first stage in β -oxidation there would still remain the question as to the immediate form in which the two carbon atoms are removed. The carbon dioxide in which they ultimately appear is probably merely the end-product of a succession of changes, but we have at present no certain knowledge as to what these changes may be.

There is, however, a further way in which fatty acids are oxidised in the body. It is found that if a fatty acid with a fairly long chain is administered there are excreted certain amounts of the dicarboxylic acid formed by the oxidation to a $\cdot\text{COOH}$ group of the $\cdot\text{CH}_3$ group at the far end of the fatty acid chain. This is called ω -oxidation because here it is the last carbon group of the chain that is attacked. It has been shown to occur with the fatty

acids containing respectively 9, 10, 11 and 12 carbon atoms, but significantly enough, not with the unfortunate one with 13 carbon atoms. For example, thus:—



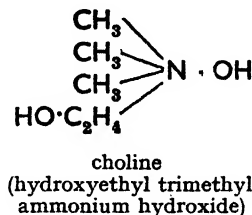
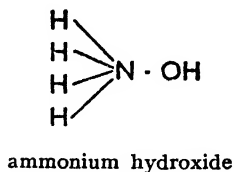
The resulting product of oxidation has now a carboxyl group at each end of the molecule, and as might be expected, is found to be oxidised in the β -position with respect to each of these carboxyl groups, forming a new dicarboxylic acid with 4 less carbon atoms. This is dangerously like burning the candle at both ends!

Before leaving this subject of fat metabolism we should call attention to the significant fact that all the fatty acids that occur in living organisms and therefore in food-stuffs contain an *even* total number of carbon atoms. For example, stearic acid with 18, and palmitic acid with 16 carbon atoms are, as we have seen, common constituents of animal fats; but the 17-carbon **margaric acid** (which does *not* occur in margarine) is a product of the organic chemical laboratory. This circumstance suggests that not only are the fatty acids broken down two carbon atoms at a time, but they must also be built up two carbon atoms at a time during the synthesis of fat in the tissues.

And recent work has confirmed this idea. Without entering into details, we might mention that it is probable that long-chained fatty acid molecules are formed in the body by the condensation of aldehydes with pyruvic acid. We shall see later (pp. 193, 200) that pyruvic acid, and, possibly aldehyde, are intermediate products in the metabolism of carbohydrates, so this gives us a clue to the method of formation of fat from the carbohydrates of the diet—a process to which we have already referred.

LECITHIN—THE PHOSPHOLIPIDES

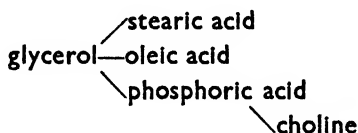
Fat as ordinarily extracted from tissues usually contains a certain proportion of substances that are more complex in structure than the simple glycerides with which we have so far dealt. One such substance is the phosphorus-containing compound **lecithin**. This has a widespread distribution in practically all cells and tissues, from which it can be extracted with ether or alcohol and then precipitated by acetone. It was first obtained in this way from yolk of egg, and from this fact received its name (Gr. *λέκιθος* = yolk of egg). The chemical structure of lecithin is fairly well known, for the substance is readily hydrolysed on boiling with dilute alkalis. In this it resembles the true fats. And further, the products are one molecule of glycerol, one each of stearic and oleic acids, and in addition a molecule of phosphoric acid and one of a basic substance called **choline**. This last-named base is a simple substance whose constitution is well known. It is a substituted ammonium hydroxide in which three of the four hydrogen atoms are replaced by methyl groups and the remaining one by a hydroxyethyl group:—



It is supposed that in the molecule of lecithin the stearic and oleic acids are combined with two of the alcohol groups of the glycerol molecule, while the phosphoric

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acid is combined with the third. But phosphoric acid being a polybasic acid, has also a valency by means of which it can unite with the choline thus :—



The fundamental resemblance between this structure and that of a simple fat is at once seen.

This lecithin is but one member of a group of similarly constituted substances that differ in the details of the fatty acids and bases they contain. As they all contain phosphorus they are referred to as the **phospholipides**. Less well-defined phospholipides have been isolated from the brain ; they occur also in other organs, such as heart, liver and kidney.

These phospholipides are of some considerable importance in the body. They seem to represent the form in which a large part of the fat is transported in the blood. That they are used for the formation of milk fat in the mammary gland is shown by the increase in the amount of *inorganic* phosphoric acid in the blood as it passes through the active gland. All tissues contain their characteristic phospholipides, and these probably play a great part in determining the relation of the protoplasm to water and its permeability to dissolved substances. The behaviour of lecithin itself to water is interesting. Its highly polar molecule may be said to be very soluble in water at one end (the phosphoric acid-choline portion), and to be quite insoluble at the other (the fatty acid end). The actual behaviour of the substance is a kind of compromise between these two extremes : it is not in the

strict sense soluble in water, but it spontaneously forms a stable emulsion with it. It is interesting to note that the factor **thrombokinas**e involved in the clotting of blood turns out to be identical with a previously known phospholipide **kephalin** which differs from lecithin only in possessing amino-ethyl alcohol, $\text{NH}_2 \cdot \text{C}_2\text{H}_4\text{OH}$, instead of choline as its basic constituent.

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CHAPTER IX

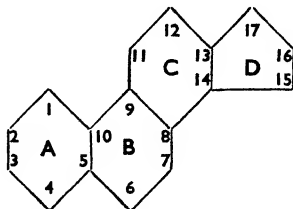
STEROLS AND STEROIDS

"The curious and varied types of substances found associated in the formerly much neglected fraction—commonly about one per cent.—which is termed unsaponifiable."—*Drummond*.

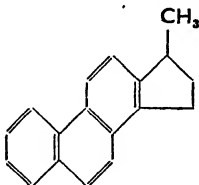
FROM what we have said in the preceding chapter it might be supposed that a lump of "fat" consists simply of glycerides of fatty acids with a small admixture of phospholipides. But all these substances, as we have seen, are easily and completely hydrolysable by boiling with alkali, whereas if a piece of ordinary tissue fat is so treated there remains a variable amount of unsaponifiable residue made up of substances of an entirely different nature. Of these the chief is **cholesterol**, a substance which, like the glycerides, is soluble in the ordinary fat solvents and crystallises therefrom in colourless needles; but from aqueous alcohol it forms flat rhombic plates which usually have one corner broken out in a characteristic fashion. In some cases the proportion of this cholesterol in the fatty components of a tissue is by no means small. Even the best brains contain a high percentage of water, but *dried* brain contains about one-sixth of its weight of cholesterol (mainly in the fatty sheaths of the medullated nerve fibres) so that the easiest way of obtaining cholesterol is to extract it by means of acetone from sheep's brains dried by mixing with plaster of Paris. It was first obtained, however, by Chevreul in the early part of the last century by extracting gall stones in a similar way — hence its name (Gr. $\chiολή$ = bile, $\sigmaτερεός$ = solid). Cholesterol gives a number of colour reactions by means of which it may be identified. For example, a solution of it in chloroform treated with a little acetic

anhydride and then strong sulphuric acid drop by drop gives a violet colouration rapidly changing to green. It is also characteristic that crystals of cholesterol irrigated on a microscope slide with strong sulphuric acid turn red at their edges; the addition of a little iodine solution then causes the crystals to turn dark violet, and eventually black.

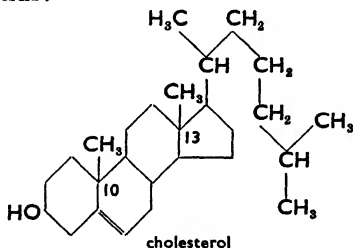
With regard to its systematic chemical properties, cholesterol behaves as a monatomic secondary alcohol of the formula $C_{27}H_{45}OH$, and as such forms esters. Indeed the cholesterol of fatty tissues is by no means exclusively in the free condition: its esters are particularly abundant in the natural grease secreted by the sebaceous glands of the skin — hence their presence in sheep's wool, from which they are extracted under the name **lanolin**. This is a very useful fatty medium, for, unlike the glycerides, the cholesterol esters, appropriately enough, are not attacked by the lipolytic enzymes of bacteria; lanolin will therefore not turn rancid. Cholesterol, further, contains a single double bond since it readily forms a crystalline dibromide through which it may be purified. Until a few years ago our knowledge of the further details of its structure was very incomplete, but now it is firmly established that the essential skeleton of the molecule consists of four "fused" carbon atom rings, of which three (A, B, C) are six-membered and are arranged as in the hydrocarbon phenanthrene, while the fourth (D) is five-membered, thus:—



The evidence for this is that on heating with selenium (which removes all available hydrogen atoms and cuts down the side chain to a $\cdot\text{CH}_3$ group) there is produced a hydrocarbon—Diels' hydrocarbon—which has been proved by synthesis to be γ -methyl-*cyclo*-penteno-phenanthrene:—



Further investigations have elucidated the structure of the side chain, the positions of the $\cdot\text{OH}$ group and the double bond, and proved the presence of two methyl groups at 10 and 13 thus:—



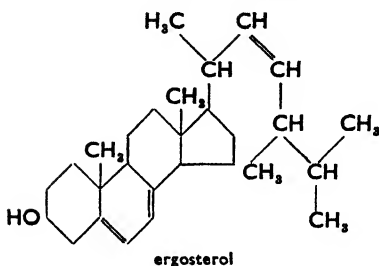
It should be emphasised that apart from the one double bond all the carbon atoms in cholesterol are saturated with hydrogen atoms so that the rings possess the properties of the paraffin rather than of the aromatic hydrocarbons.

There is no doubt that cholesterol is synthesised by the animal body, for the cholesterol content of the eggs laid by hens during an observation period has been found to be much greater than their simultaneous intake of the substance in food. But we do not know from what precursor

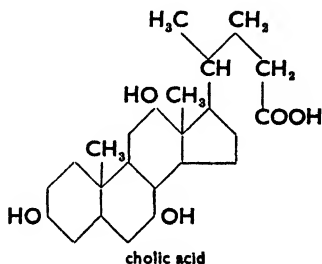
this synthesis starts. It is, however, significant, that the carbon chain of such an acid as oleic contains 17 carbon atoms together with a carboxyl group, and it is, of course, easy on paper so to write its formula as to bring these carbon atoms into the same relative positions as those of the complex ring system of cholesterol. Such a speculation is, however, at best only a stimulus and no substitute for the experimental evidence which we at present lack.

Nor can we lay claim to any but fragmentary ideas as to the functions subserved by cholesterol as a constituent of living matter. It seems to be particularly concentrated in cell membranes such as the envelopes of red blood corpuscles, and from this and the fact that a tissue's power of imbibing water is the greater the more cholesterol it contains we may deduce that cholesterol shares with the phospholipides the function of regulating the uptake and transmission of water and dissolved substances in solution. That cholesterol is important to tissues is in any case suggested by the fact that it is retained with great tenacity during starvation.

So far, in considering cholesterol we have been dealing with one of a number of closely related substances, all containing the same fundamental ring structure and known collectively as the **sterols**. They are found in the un-saponifiable residue not only of animal but also of plant tissues. It must suffice here to mention but one other of them — **ergosterol** — which not so long ago was a mere chemical curiosity isolated from fungus-infested rye (ergot) but is now a substance of first-class biochemical importance on account of its relation to vitamin D. It differs from cholesterol in possessing two double bonds in its ring skeleton and a third in its slightly more complex side chain:—



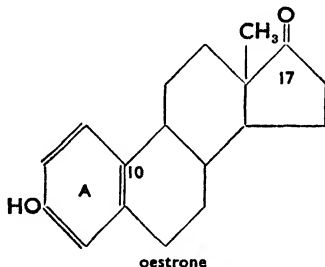
Another group of substances possessing this same sterol skeleton are the bile acids whose derivatives play such an important rôle in the digestion and absorption of fats (p. 129). There are a number of these in various animals' bile, but the mother substance of them all is **cholic acid**. Here we have a completely saturated skeleton, three $\cdot\text{OH}$ groups, and a simple side chain ending in a $\cdot\text{COOH}$ group:—



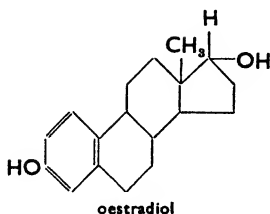
Linked, amide-fashion, with the $\cdot\text{NH}_2$ group of taurine or glycine it forms respectively the taurocholic and glycocholic acids present in the bile of most mammals. It is tempting to suppose that cholic acid is formed in the body from cholesterol which accompanies it in the bile. But the administration of cholesterol does not increase the output of cholic acid, and even other more

closely related sterols seem merely to stimulate, but not to act as raw materials for, the formation of bile salts.

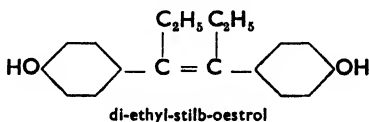
Of recent years a great deal of progress has been made with the isolation and investigation of some of the least abundant constituents of living tissues. Of this we shall meet other striking examples later, but for the moment the sex hormones responsible for the bodily changes associated with reproductive processes call for attention as they have turned out also to possess the sterol skeleton as the essential framework of their chemical structure. The first of these to be isolated was the oestrus-producing hormone **oestrone** which is secreted (albeit largely in a combined form, and accompanied by other, inactive, sterols) in the urine of women during pregnancy. Its constitution is represented by the formula:—



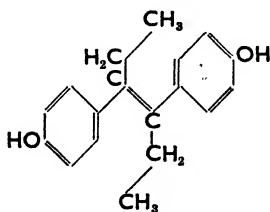
in which it will be noticed that ring A is in this case completely aromatic (i.e. unsaturated) and therefore cannot carry a methyl group at 10, while the side chain at 17 consists merely of a doubly-linked oxygen atom. But the actual more potent hormone as extracted from the ovary itself (4 tons of pigs' ovaries yielded 0.025 grm. of it!) is not a ketone but the corresponding secondary alcohol **oestradiol** :—



We might mention in passing that the power of stimulating uterine development, cornification of the vaginal epithelium in ovariectomised mice, and other feminine activities, is by no means confined to these naturally occurring substances. Many synthetic oestrogens are known, of which the most potent are the derivatives of di-phenyl-ethylene (stilbene), for example:—



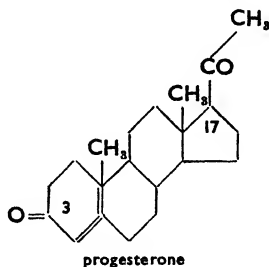
which may also be written:



When conception has taken place the corpus luteum liberates the hormone **progesterone**, which, among other functions, sensitises the uterine mucous membrane for the

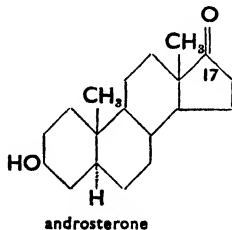
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embedding of the fertilised ovum and helps in the development of the mammary glands. Its constitution is:—

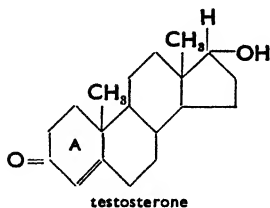


— it has an oxygen atom instead of the usual $\cdot\text{OH}$ group in position 3, but it does possess a side chain of two carbon atoms at 17.

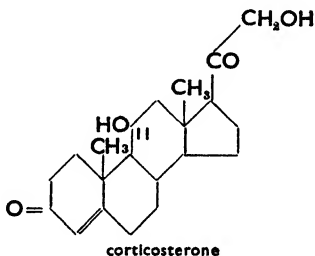
Corresponding male hormones are also known. **Androsterone** was, like oestrone, first isolated from urine (male, of course, in this case), and was found similarly to possess a keto-linked oxygen atom as side chain at 17. But it has no double bond:—



Also, as in the case of the female hormone, the corresponding secondary alcohol is several times as potent as the ketone, while **testosterone** extracted directly from testis tissue and found to possess a double bond in ring A, is the most potent of all in stimulating comb growth in castrated cocks and other manifestations of maleness:—



Still more recently the secretions of the life-essential suprarenal cortex have been investigated. From this tissue some 15 different sterol derivatives have been isolated, of which the chief is **corticosterone**:—



The structure of this will be seen to be very similar to that of progesterone, but we have here a new departure in the form of an $\cdot\text{OH}$ group in position 11. Curiously enough, however, this seems to hinder the action of the hormone, for the substance obtained by removing this $\cdot\text{OH}$ group is some ten times as active as corticosterone itself in prolonging the lives of decorticated rats. But neither this nor any of its accompanying sterol derivatives can be said to be the one life-giving principle of the suprarenal cortex, capable of prolonging indefinitely the lives of animals deprived of their natural source of it.

With this we come to the end of this all too scanty survey of the sterols and related compounds — substances that in a

remarkably short space of time have established so powerful a claim to significance as essential constituents of living matter as now to merit a chapter to themselves. If what we have said here about the sterols proper, the bile acids and the sex hormones, together with what follows later with regard to antirachitic vitamins, were not sufficient amply to justify this claim we could go on to mention that many other substances, outside our present scope but equally significant — the essential components of the **saponins** and of heart drugs such as **digitalin** — are also sterol derivatives, as is also possibly the evocator of embryological development; while not very far removed from them are the inimical carcinogenic (cancer-producing) hydrocarbons. This is certainly a case where it would seem that Nature has slightly modified the same piece of biochemical machinery in order to adapt it for a wide range of different uses. Innumerable problems arise as to how these apparently slight modifications of chemical structure suffice to produce such diverse effects in living tissues — problems whose solution will require a knowledge not only of the structure of the active substance but of the architecture of the living matter on which it acts. When we possess this we may be able to give a rational explanation of the circumstance that the world of difference between maleness and femaleness depends on the presence or absence of nothing more romantic than an extra hydroxyl or methyl group, or a double bond more or less, associated with the same all-pervading sterol skeleton!

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CHAPTER X

THE CHEMISTRY OF THE CARBOHYDRATES : THE DIGESTION OF STARCH AND SUGAR

"The chemistry of the carbohydrates has become a problem in geometrical permutations, and almost all the possible permutations have been identified, and most of these synthesised."—*Leathes*.

FROM the point of view of animal physiology or medicine the chief substances included under the term carbohydrates are the sugars and the starches. In these as in the other members of the group the molecule contains two atoms of hydrogen to each one of oxygen, that is, the proportion in which these elements occur in the carbohydrate molecule is the same as that in which they occur in water, so that the ultimate composition of any carbohydrate could be represented as being made up of a certain number of carbon atoms together with a certain number of water molecules—hence the group name (which, however, is intended to express merely this fact of ultimate composition and to imply nothing as to the mode of combination of the hydrogen and oxygen atoms within the chemical structure of the molecule). It should be pointed out that by no means every substance in whose molecule the hydrogen and oxygen atoms occur in the ratio of two to one is included in the group of carbohydrates: lactic acid, for example, although its formula adds up to $C_3H_6O_3$, quite obviously does not belong here—in fact, the term carbohydrates is restricted to the sugars and their condensation products. On this account it has been proposed to substitute the name **glucides**

for the, to this extent, indefinite term carbohydrates, but up to the present the suggestion has not been generally adopted.

As the sugars are the simpler carbohydrates we will study them first. Everybody knows the general properties of a sugar. It is usually a colourless crystalline substance readily soluble in water and possessing a more or less well-marked sweet taste. **Cane sugar** is the most familiar though by no means the most typical member of the group. The sugars fall naturally into two main groups—simple sugars (monosaccharides) and compound sugars (disaccharides) which are condensation products of two monosaccharide units. The commonest monosaccharides such as **glucose*** and **fructose*** possess six carbon atoms in the molecule and the total formula $C_6H_{12}O_6$: these are called hexoses (Gr. ἑξ=six). Monosaccharides with five carbon atoms and a total formula $C_5H_{10}O_5$ are also known, but apart from their occurrence in the molecule of nucleic acid these pentoses do not figure very prominently in animal metabolism. Of the compound sugars the commonest are the disaccharides cane sugar (**sucrose**), **maltose**, the sugar of malt, and **lactose**, the sugar of milk (Lat. *lac*, *lactis*=milk). Each of these substances may be regarded as being formed by the condensation of two monosaccharide molecules with the elimination of a molecule of water between them so that the total formula will in each case be $C_{12}H_{22}O_{11}$. This condensation is readily reversed by boiling the disaccharides with a little dilute acid when they are

* Glucose is sometimes known as **dextrose**, or yet again as **grape sugar**. It is important for the student to realise that these three names refer to one and the same substance. In a similar way **lævulose** and **fruit sugar** are alternative names for fructose.

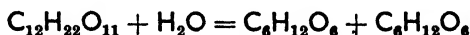
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rapidly hydrolysed into their constituent monosaccharides. Under these circumstances

cane sugar	yields	1 mol. glucose + 1 mol. fructose
maltose	„	1 mol. glucose + 1 mol. glucose
and lactose	„	1 mol. glucose + 1 mol. galactose —

a hexose we have not previously mentioned, and for the naming of which the Greek word for milk (*γάλα, γάλακτος*) is called upon, the Latin having been used in naming the parent disaccharide.

Of course the general equation representing each of these hydrolyses is



and it will be noticed that each of the disaccharides we have mentioned yields at least one molecule of glucose on hydrolysis; they are all compounds of glucose and so are included in the general group of the **glucosides**.

Passing over a number of somewhat more complex but, from our point of view, unimportant carbohydrates, we now arrive at the most complex members of the group whose molecules are built up of an enormous number of monosaccharide units. Among these complex carbohydrates we are interested in the **starches** and, to a certain extent, in the **cellulose** which forms the material of the cell walls of plant tissues. They are grouped together as the **polysaccharides**, and as the linking of the monosaccharide molecules will in general involve the loss of one molecule of water from each hexose molecule their general formula tends to approach $(\text{C}_6\text{H}_{10}\text{O}_5)_n$ where n is a number of the order of several thousands.

In fact so large is the aggregate of monosaccharide units constituting the chemical structure of the polysaccharides that the concept of molecules hardly applies to these substances.

Modern X-ray analysis, which has shown that in a crystal of common salt the sodium and chlorine atoms are all equally spaced in a kind of lattice so that no particular atom belongs any more to one of its neighbours than to another, has shown also that in polysaccharides—cellulose is the one that has been studied most extensively—the monosaccharide units are arranged in a long chain, and one feels that if it were possible to cut this chain by means of a knife each portion would be just as much entitled to the name cellulose as the original complex, although the knife would have separated fragments that in the whole structure were united by chemical forces. This is, of course, hardly what one would expect to be able to do with a molecule as defined in a strict chemical sense, and is what we meant when we said that for substances of such complex constitution as the polysaccharides, and, for that matter, other similarly complex substances such as proteins and the highly-polymerised hydrocarbon india-rubber, the concept of molecule ceases to apply.

This, then, is a brief survey of the various substances included in this group of the carbohydrates. We must now go on to consider their several chemical properties in more detail. Dealing first with the sugars, we should mention that one of the most characteristic properties possessed by most sugars is the power of acting as reducing agents. This reducing property is made use of in the commonest test for sugars, namely the production of a red precipitate of cuprous oxide when they are boiled

with the mixture of cupric sulphate, caustic soda and potassium sodium tartrate known as **Fehling's solution**. This property of reducing Fehling's solution is shown by glucose and fructose and also lactose and maltose; but in general the disaccharides are less powerful reducing agents, i.e. will reduce a smaller quantity of copper than the monosaccharides in equal concentration, and there are some disaccharides, notably *cane sugar*, that will *not* reduce Fehling's solution at all. The reasons for these differences will be clear when we have compared the detailed chemical structure of these substances later, but even at present it will be evident that the fact that a sample of urine reduces Fehling's solution must not necessarily be interpreted as indicating that glucose is the reducing substance present, and, without further tests, be made the basis of a diagnosis of sugar diabetes: the reducing sugar present might well be lactose, as indeed is often the case when the female body is producing large quantities of lactose during lactation.

Meanwhile we might add that an acid solution of copper salts is more difficult to reduce than an alkaline one such as Fehling's solution is. Thus it comes about that a solution of cupric acetate in acetic acid (**Barfoed's reagent**) is reduced, at all events readily, only by monosaccharides, but not by the less powerfully reducing disaccharides, and so can be used for distinguishing the two groups of sugars. There is probably little need to suggest to the student the way in which these facts can be applied to the qualitative analysis of solutions containing sugars. We will mention only that a reducing sugar can be removed from the presence of cane sugar by careful oxidation in boiling solution with Fehling's solution until a slight permanent blue colour indicates

excess. If now the cuprous oxide be filtered off the cane sugar remains unchanged in the filtrate. It can be tested for by acidifying with strong hydrochloric acid and boiling the solution, when the cane sugar is rapidly hydrolysed to an equimolecular mixture of glucose and fructose (which will, of course, after appropriate neutralisation, now reduce Fehling's solution); or by heating it with a large excess of strong hydrochloric acid and a little of a solution of α -naphthol. Under these circumstances the fructose first liberated by hydrolysis is further converted by the strong acid into a ring compound—furfuraldehyde—and this then condenses with the α -naphthol to give an immediate purple coloration. It should be mentioned that a similar reaction can be obtained with other sugars such as glucose, but only after long boiling: it is only fructose, or cane sugar, which yields fructose on hydrolysis, that gives a conspicuous colour by the time the mixture is really boiling.

Apart from this α -naphthol test for fructose we have practically no specific colour reactions for the other individual sugars. When therefore a reducing sugar has been found to be present in some material other means have to be adopted for its identification.

Now when a chemist has discovered the *group* to which an organic substance belongs he usually decides which particular member of that group he is dealing with by determining its melting- or boiling-point. In the case where a substance will not even melt without decomposition it is usual to convert it into some crystalline derivative whose melting-point is sharp and definite. The sugars are such substances, but they form with the organic base **phenyl-hydrazine**, yellow crystalline compounds called **osazones**, and these osazones have definite

crystalline forms and melting-points by means of which they, and so their parent sugars, can be identified. Hydrazine itself is a compound of two amino groups:— $\text{NH}_2 \cdot \text{NH}_2$: its phenyl derivative is $\text{C}_6\text{H}_5 \cdot \text{NH} \cdot \text{NH}_2$. In practice, in order to make the osazone of a sugar, one takes, say, half a test-tubeful of a one per cent. solution of the sugar, adds as much phenylhydrazine hydrochloride as will lie on a sixpenny piece and as much sodium acetate as will lie on a shilling. This last-mentioned salt acts as a "buffer" (see p. 397) and prevents the hydrochloric acid liberated as the phenylhydrazine is used up from producing too great an increase of acidity, for this would hinder the reaction. The mixture is warmed until everything is in solution, filtered if not clear, and then heated in a boiling water-bath for half an hour. By the end of that time the osazone will have been formed, and if it is a relatively insoluble one like that of glucose (phenylglucosazone) it will have already crystallised out from the hot liquid. If, however, it is more soluble, as in the case of lactosazone or maltosazone, it will only crystallise out on allowing the tube to remain undisturbed in the water-bath until the whole is cool. The osazones of the various sugars have different crystalline shapes and habits by which they can be readily identified under the microscope. Glucosazone crystallises in long yellow needles arranged in "sheaves," lactosazone forms roundish masses of small needles that suggest "hedgehogs," while the crystals of maltosazone have a distinct breadth, i.e. they consist of tablets that usually arrange themselves in "rosettes." In order to make the identification complete this observation of the crystalline form should be followed by a determination of the melting-point of the osazone, for which purpose it should first be purified

by recrystallisation from dilute alcohol. The melting-points of the osazones most likely to be met with in biochemical work are as follows :—

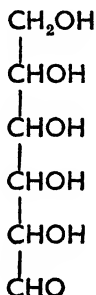
phenylglucosazone	204–205° C.
phenyllactosazone	200° C.
phenylmaltosazone	206° C.

With regard further to these osazones it should be mentioned that the osazone obtained from fructose is identical with phenyl*glucosazone*, the reason being that the molecules of fructose and glucose differ only in the two groups that are attacked by the phenylhydrazine, and that during the formation of the osazone the difference in structure between these two groups is obliterated. Then, again, cane sugar does not form an osazone at all because, as we shall see later in more detail, the linkage between the glucose and fructose components of its molecule involves those particular reactive groups on which the possession of reducing properties and the power of forming an osazone depend.

This is perhaps the most convenient point at which to remind the student that the reducing action of sugars on copper solutions is utilised not only for the detection of sugars but also for their estimation. If, however, a sugar solution is titrated directly into a known volume of a boiling standardised Fehling's solution the point at which the last trace of blue colour disappears is very difficult to observe on account of the presence of the red cuprous oxide precipitate. Various methods have been adopted for eliminating this difficulty. Pavy added ammonia, which dissolves cuprous oxide to a colourless solution, but is very liable to boil away during the titration. Benedict has proposed a copper solution

containing potassium thiocyanate so that a *white* precipitate of cuprous thiocyanate is obtained, and more recently Lane and Enyon have worked out the simple procedure of adding to the Fehling's solution towards the end of the titration a little methylene blue. This is also reduced—to a colourless compound—by sugar, so that at the end-point a very considerable loss of colour takes place, producing a change that is easily observable even in the presence of the red precipitate. In Hagedorn and Jensen's modern method for the estimation of sugar in blood the use of copper solutions is avoided altogether and the sugar is estimated by its power of reducing potassium ferri- to ferro-cyanide. The amount of ferricyanide left unreduced out of the excess taken is determined by adding potassium iodide when iodine is liberated and is titrated in the usual way with standard thiosulphate. Since ferricyanide solution is not stable it must be standardised each day in the same way against the thiosulphate solution. Thus the sugar estimation ultimately resolves itself into an ordinary iodometric titration. It is perhaps hardly necessary to mention that cane sugar can also be estimated by these methods by first hydrolysing to a reducing mixture of glucose and fructose. From all this it will be seen that a very great deal can be done in the detection, identification and estimation of the commoner sugars with simply a knowledge of a few significant points in their chemical behaviour, but in order to understand the differences in their behaviour and their relationships to the general group of which they are individual members it is necessary to possess a much more penetrating knowledge of the details of their chemical structures. To this topic we must now address ourselves.

Considering first the commonest hexose, glucose. It is merely a matter of the most straightforward analysis to show that its total formula is $C_6H_{12}O_6$, and a relatively simple problem to show that it contains a "potential" aldehyde group and 5 $\cdot OH$ groups, and that it may be regarded as a derivative of the straight chain paraffin hydrocarbon hexane C_6H_{14} . This would give us a tentative formula as follows :—



But with regard to the completeness with which such a formula represents the properties and structure of glucose several questions at once arise. Of these the first is that concerning the relative arrangement in three dimensions of the groups we have thus arbitrarily represented in two. The chemical properties of a substance depend, of course, on the groups of which it is composed and also on the relative spatial positions of these groups; but if these groups happen not to be particularly reactive the differences of chemical properties produced by differences in their space arrangement are apt to be few, and these relatively inconspicuous. There is, however, one *physical* property of a molecule that is affected by even the smallest change in the space arrangement of its atoms or groups, and that is its effect on light, manifested in the

phenomenon of **optical activity**. As this is a matter of fundamental importance in sugar chemistry we shall give a brief outline of it.

It is necessary first to be to some extent familiar with the nature of light. In terms of classical theory light is a form of energy composed of transverse waves in the all-pervading ether. This means that each ether particle oscillates in a plane which is at right angles to the direction in which the light is travelling. In this respect the light waves resemble waves on the surface of water, where it is a matter of observation that a floating particle moves up

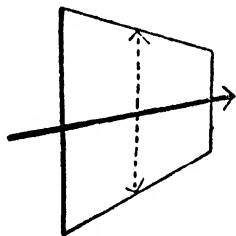


FIG. 8.

and down at right angles to the general surface, while the waves move forward along the surface. (On the other hand, in sound waves we have a case where the movement of the vibrating air particles is to and fro in the same direction as that in which the sound is progressing.) Now it is evident that there is an infinite number of planes at right angles to the direction in which a ray of light may be travelling. For example, in Fig. 8 let the thick arrow represent the direction of a ray of light travelling downwards away from the reader; then one possible plane at right angles to this direction is that shown, and a given

ether particle might be vibrating up and down in the dotted line in the plane. On the other hand, an alternative arrangement is that shown in Fig. 9, where the ether particle vibrates from side to side: and between these two extremes any number of possible intermediate conditions exist. Now, ordinary light — even that of one colour — consists of a mixture of rays in which the vibrations of the ether particles are in all these different planes at right angles to the direction of the rays. It is possible, however — most easily by passing the light through certain crystals, of which Iceland spar is the best known — to filter off all the rays except those in which the vibration

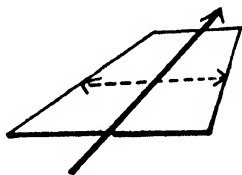


FIG. 9.

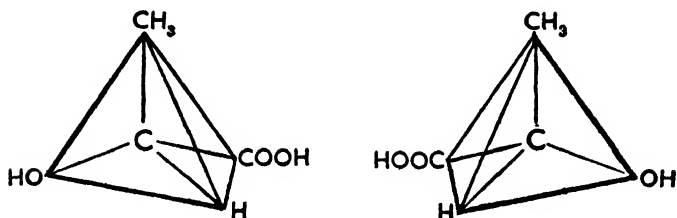
is in *one* particular plane. These remaining rays, in which, then, all the ether particles are vibrating in the same plane, form a beam of what is called polarised light. No difference is noticed between this light and ordinary daylight when they fall on the eye, but they differ with regard to its behaviour when passed through a crystalline structure, for example, a second crystal of Iceland spar. For, as we have said, it is a property of such crystals as Iceland spar to let through only such rays as are vibrating in one particular plane which has a definite relation to the axes of the crystal, and is known as its optical plane.

If, then, we have a ray of this polarised light, it will pass through the crystal, when the optical plane of the crystal corresponds with the actual plane in which the vibrations are occurring, but it will not get through the crystal if its optical plane is turned at right angles to the plane of vibration of the light. In intermediate relative positions of the two planes more or less light gets through according as they are nearly coincident or more nearly at right angles. Of ordinary daylight, of course some will be transmitted in any position of the crystal, because there will always be some rays that are vibrating in the optical plane. Now it has been known for a long time that many substances possess the power of changing the plane of polarisation of a beam of polarised light passing through them or their solutions. In other words, the beam of light after emergence from the substance or solution has a different plane of vibration from that it possessed when it entered. Such substances are said to be optically active or to exhibit the phenomenon of optical rotation. The angle through which the plane of vibration is turned depends on the particular substance used, on the strength of the solution, and the length of solution through which the ray has passed. Now suppose that we have a source of light polarised in one particular plane, and view it through a crystal of spar so arranged that its optical plane corresponds to the plane of vibration, the light will pass through in maximum amount. Let us now interpose between the source of light and the crystal a layer of some optically active substance: the plane of vibration of the light will be rotated so that when the ray emerges its plane will no longer coincide with the optical plane of the crystal. The intensity of light seen through the crystal will, therefore, be diminished. The

maximum intensity can, however, be restored by turning the crystal until its optical plane corresponds with the new plane of vibration. It will be seen that the angle through which the crystal will need to be turned will be exactly equal to that through which the optically active substance has turned the plane of polarisation. In this way we can measure degrees of optical activity, the optical rotation of a substance being defined as the angle through which the plane of polarisation of light would be turned by passage through 10 cm. of a 100 per cent. solution of the substance (of course the observation is usually made on a much more dilute solution and the result multiplied up). Now it has long been known that of any substance that is optically active two forms always exist : one of these forms rotates the plane of polarisation of light to the right (dextro-rotatory), while the other rotates it to an exactly equal extent to the left (lævo-rotatory). Thus, the lactic acid that forms a normal constituent of muscle tissue is dextro-rotatory or *d*-lactic acid, while that produced by the bacterial decomposition of sugars is lævo-rotatory or *l*-lactic acid. Similarly *l* and *d* forms of tartaric acid and of many other substances are known. A substance prepared synthetically in the laboratory is always optically inactive : it consists of a mixture of the two forms in exactly equal proportions so that the right-handed rotation produced by the *d* isomer is exactly neutralised by the left-handed rotation due to the *l* form ; ordinary lactic acid obtained from sour milk is a mixture of this kind. The two active forms of a substance are usually practically identical in chemical behaviour, differing only in such properties as solubility or crystalline form. The most beautiful example of this is furnished by the tartrates. It was

Pasteur who showed, in one of his early investigations, that the crystals of the sodium ammonium salt of ordinary inactive tartaric acid can be sorted into two groups whose shapes bear to one another the same relationship as those of, say, the right and left hands. A crystal of one type is not superimposable upon but is, as it were, the mirror image of a crystal of the other. The crystals are "right-handed" and "left-handed"—enantiomorphous, if it is necessary to say it in Greek. And the great point is that when these right-handed and left-handed crystals are dissolved separately in water the one solution turns the plane of polarisation to the right and the other to the left. It is evident that this optical activity is due to some kind of "lopsidedness" of the molecule, which shows itself not only by the difference in the effect on polarised light, but also in the crystalline form. When it was further considered it became evident that such a lopsidedness could arise in an organic molecule if the four valencies of the carbon atom are distributed equally in space and each happens to be attached to a different group. The carbon atom is imagined as being situated at the central point of a regular tetrahedron (triangular pyramid) with the valencies directed one to each of the four corners, where the other groups of the molecule are supposed to be attached. In the case where these groups are all different it requires but a little clear thinking to show that by a mere interchanging of the positions of any two of the groups it is possible to obtain an alternative arrangement which cannot in any way be superimposed on the first but is its mirror image. We can illustrate this best by considering one of the simplest cases, namely that of lactic acid, $\text{CH}_3\cdot\text{CHOH}\cdot\text{COOH}$. Arranging its groups in the three planes of space we have the two

mirror-image forms obtained by interchanging the positions of the $\cdot\text{H}$ and $\cdot\text{COOH}$ groups thus :—

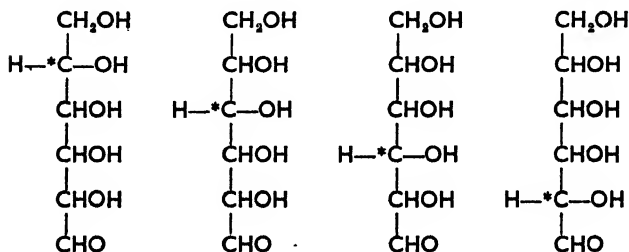


and if the reader will imagine these formulæ as solid objects that he can rotate he will soon realise that it is impossible to turn the second into any position in which it exactly coincides group for group with the first. The most satisfactory way of convincing oneself of this is to make little models of the molecule out of match-sticks and fragments of cork or plasticine. Each of these formulæ is evidently that of a lactic acid ; owing to the presence of the same groups in the molecule the main chemical properties would be practically identical in the two cases, but, nevertheless, owing to the circumstance that the two arrangements are not absolutely identical we should expect small differences of detail between the properties of the two forms, and the fact that the one arrangement is the mirror image of the other would well account for the opposite effects of the two forms on polarised light. In this way we account for the existence of the lævo- and dextro-rotatory forms of the acid and of the optically inactive acid which is a mixture of equal quantities of these two forms.

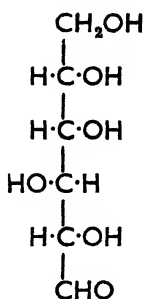
By this time the reader is probably wondering why we have treated of this matter of optical activity in so

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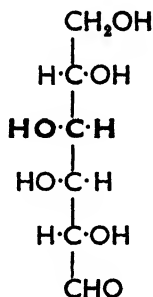
much detail. The reason is that in the sugars we have more abundant opportunities for the occurrence of optical isomerism than in any other groups of substances. A little consideration will show that in the formula for the simplest hexoses there are no fewer than four carbon atoms that are attached to different groups and that by their asymmetry can therefore *each* give rise to *two* optical isomers:—



And since, further, each arrangement of groups around any one carbon atom may be associated with either one or the other of the two possible arrangements around any other carbon atom the total number of possible optical isomers of this simple hexose molecule will be $2 \times 2 \times 2 \times 2 = 16$. Now each of these will be a different sugar, and, as may well be imagined, it absorbed a considerable portion of Emil Fischer's energies to decide exactly which particular molecular architecture was possessed by each of these sixteen hexoses. Of course, of these only one is glucose, and this turns out to be the one that has the groups arranged around the asymmetric carbon atoms in the way shown in the following formula:—

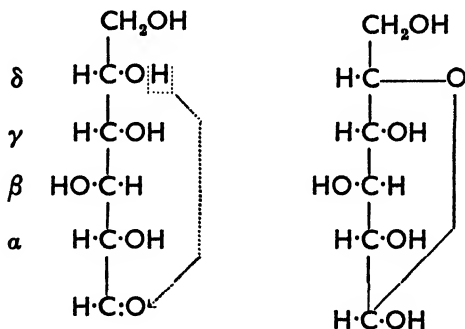


In passing it is interesting to note that galactose (p. 156) differs from glucose only in the arrangement of one link in the chain, thus:—



But although we have thus represented the space distribution of the groups around the carbon atoms we must hasten to point out that even so the formula given above does not accurately and completely represent the properties and behaviour of glucose. For one thing a polyhydroxy-aldehyde such as we have represented would be expected to be a very powerful reducing agent, whereas the reducing powers of glucose are not as striking as all that. It reduces Fehling's solution, but only on boiling;

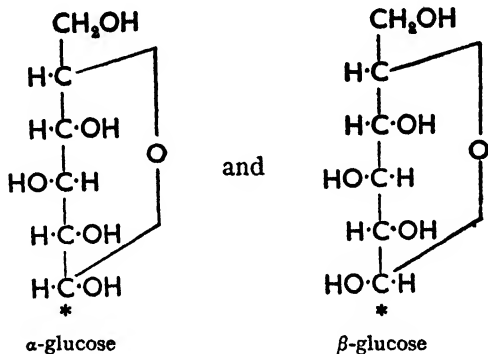
it is not oxidised by atmospheric oxygen—and so on. For these reasons it is supposed that in glucose the aldehyde group is only “potential,” that is, it is masked by having reacted with one of the hydroxyl groups, receiving therefrom a hydrogen atom and so forming a ring structure in which an oxygen atom takes part. The question then arises as to which of the hydroxyl groups is in this way involved. For a long time it was thought to be that in the γ position to the aldehyde group, but this was little more than a guess, and now recently, as a result of intricate but fascinating chemical studies, it has been proved that the “oxygen bridge” connects the aldehyde group with the δ carbon atom. We thus arrive at the present-day formula for glucose:—



Now it will be noticed that in this arrangement the carbon atom of what was the aldehyde group has become asymmetric; it is now attached to four entirely different groups—and as a result there arises the possibility of two optical isomers of glucose which, possessing mirror image structures should possess different optical rotatory powers. As a matter of fact two such forms of glucose

are known, and their existence affords very powerful support to the idea of the structure of the glucose molecule that we have just expounded. These two forms of glucose, which are both dextro-rotatory, but one to a greater extent than the other, are termed α - and β -glucose respectively.

They will be represented by the formulæ

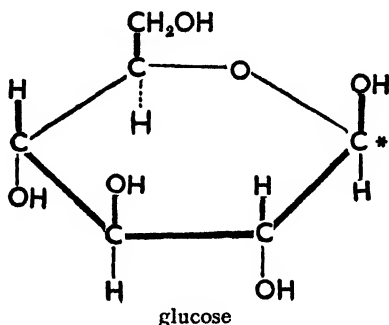


respectively, which differ merely in the orientation of the lowermost group of the chain. Ordinary glucose, at all events when in solution, is regarded as an equilibrium mixture of these substances.

In writing these formulæ we have represented the oxygen bridge as being in a plane behind the main carbon chain. This was to show the relation between these formulæ and those previously given for glucose. But all we know about the arrangement of atoms in ring structures would lead us to believe that in the actual molecule the one oxygen and five carbon atoms composing the skeleton of the glucose molecule are much more evenly spaced, so that instead of being a straight-chain compound as we

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at first represented it, glucose turns out to be a typical ring compound—a conclusion that can be emphasised by somewhat changing the proportions of the formula and writing it in the following generally accepted modern form:—

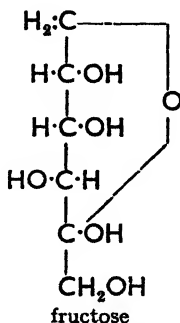


In this formula it is very important to notice that, if we assume as usual that the valencies of the carbon atoms are always rigidly directed towards the four corners of a regular tetrahedron, for ring formation to occur at all a "twist" *must* be given to the carbon atom bearing the terminal CH_2OH group, so that the H atom also attached to the same carbon atom comes to be represented *below* the plane of the molecule and not above as perhaps might have been expected. This point is very easily confirmed by means of simple models.

But this is not yet quite all there is to say about glucose, for there is known, at least as a methyl derivative, yet another modification which is so surprisingly reactive that it will reduce Fehling's solution even in the cold. As the first two letters of the Greek alphabet were already appropriated for the forms of glucose already described this active form of glucose was labelled γ -glucose, and

now that its constitution has been elucidated we know that it differs from ordinary glucose in that the oxygen bridge extends between the aldehyde group and the γ carbon atom. So that γ -glucose turns out actually to be a γ compound, which is very fortunate seeing that its naming was purely arbitrary in the first place.

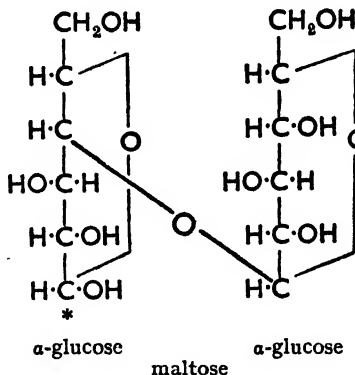
Turning now to the constitution of fructose, it will suffice to say that this sugar was long ago shewn to be a ketone of this general structure:—
 Later it was shown that the space arrangement of the :CHOH groups is the same as in the corresponding portion of the glucose molecule: in other words, the molecules of glucose and fructose differ only in their end two groups, and as the difference in these is obliterated in the formation of an osazone, it is inevitable that fructose should give the same osazone as glucose. More recently it has been proved that just as in the case of the aldehyde group of glucose the keto-group of fructose is only potential as it is involved in the formation of an oxygen bridge with the carbon atom in the δ position to itself, which is in this case the end member of the chain, thus:—



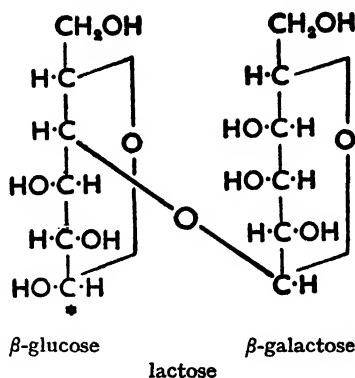
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So that ordinary fructose like glucose contains a six-membered ring of which one atom is oxygen.

We come now to consider the structure of the disaccharides. Seeing that both maltose and lactose reduce Fehling's solution, but not so powerfully, i.e. in not so large amount per given weight of sugar, as do the monosaccharides it is evident that in both of these disaccharides the linkage of the two monosaccharide constituents must involve the potential aldehyde group of one of them leaving the other free to exhibit its reducing properties and its power of forming an osazone. In the case of maltose it has further been shown that the molecule of glucose whose aldehyde group is concerned in the linkage is of the α configuration, and these together with other considerations lead us to represent the structure of maltose thus:—



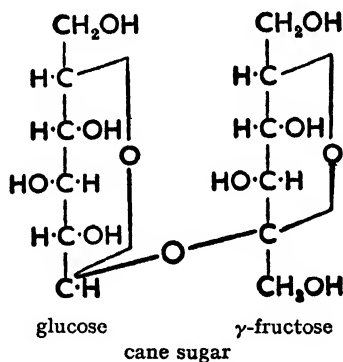
In a similar kind of way it has been shown that lactose is a compound of the β form of galactose:—



Contrasted with these reducing disaccharides we have cane sugar which will neither reduce nor form an osazone. These facts are interpreted by supposing that both the potential aldehyde group of the glucose component and the potential keto-group of the fructose component, the groups, that is, that are concerned with the formation of the oxygen bridges, are also involved in the linkage between the two monosaccharides, with the result that the rings cannot be opened up, and therefore the properties characteristic of the aldehyde and ketone groups cannot be exhibited. This, it will have been noticed, is not the case in lactose and maltose in each of which one potential reducing group (marked *) remains unaffected by the linkage. But there is this further of interest in connection with the constitution of cane sugar. Recently, but only surprisingly recently (1928), one of the possible isomers of cane sugar has been synthesised, and for this it was found necessary to use derivatives of the γ form of fructose which is analogous to γ -glucose and contains a five-membered ring. The fructose, therefore, as it exists

as a component of the cane sugar molecule is an active or γ -sugar, but since ordinary fructose obtained by the decomposition of cane sugar is the inactive δ form it is evident that the γ -fructose must on liberation by hydrolysis at once pass by intramolecular change to the inactive six-membered ring structure.

But even now, in spite of the commonness and accessibility of cane sugar, our knowledge of its structure is still in one particular incomplete, inasmuch as the method of synthesis leaves it uncertain whether either its glucose or its fructose component is of the α or the β configuration. The facts already known, however, can be expressed by a formula as follows:—



In the polysaccharides we have substances very different in general properties from the sugars. Instead of relatively simple substances, readily soluble and crystallisable, the polysaccharides are substances of high molecular weight, either insoluble or forming only colloidal solutions in water, and composed apparently of enormous numbers of monosaccharide groups united in

long chains. In ordinary vegetable **starch** these monosaccharide units appear to be molecules of glucose of the α variety, while the cellulose composing the cell walls of plant cells seems to differ in being composed of chains of molecules of β -glucose. Of these two polysaccharides starch is by far the more important from our present point of view. As is well known, it is formed in plant cells in granules composed of layers arranged round a more or less eccentrically situated hilus, the actual form of the grain being characteristic of the plant species from which the starch is obtained. Starch grains are unaffected by cold water, but in hot water they swell up and form a colloidal solution known as starch paste. This is a matter of some practical importance not only in the laundry, but also in the laboratory. For one thing it means that in analysis other soluble substances can be separated from starch by leaching out with *cold* water, whereas if hot water were used the starch would simply form a pasty mass that would tend to obscure everything. On the other hand, when starch paste is required, the technique for preparing it cleanly is to suspend the starch grains in a little cold water and to pour this suspension into the requisite amount of hot water. The starch grains do not then stick together in a mass, but swell up separately and dissolve. The starch when thus in solution shows typical colloidal properties, inasmuch as it is precipitated by salts, for example it is completely salted out by half-saturation of the solution with ammonium sulphate. The usual test for starch is the well-known dark blue coloration it gives with iodine — a coloration that fades on warming but reappears (if the heating has not been too prolonged) on cooling. When starch is warmed with a little dilute acid it is hydrolysed

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first to somewhat simpler but still colloidal substances known as **dextrins** which give a red colour with iodine, and then to molecules of maltose. Under the conditions of the experiment this maltose is usually hydrolysed to its constituent glucose molecules.

The digestion of starch in the body follows very similar lines. The starch present in food is attacked first of all by the enzyme **ptyalin** of the saliva (Gr. πτύω = I spit), and this breaks it down first into dextrins and then into maltose. The process can be easily followed in a test-tube in which some saliva is added to a little starch paste. If the mixture be kept warm the digestion proceeds rapidly, and as it progresses it will be found that as the starch becomes used up small drops of the mixture removed at intervals from the main bulk and tested with iodine give less and less of the typical blue colour; when the more complex dextrins are present it will be found that a red coloration is produced, and at the end, when only the simpler dextrins and maltose remain, the tested drop will give no colour with iodine at all. But in the mouths of even those of us who are most fastidious with regard to chewing, very little more occurs than the mixing of the starch and the saliva. The main portion of the salivary digestion occurs while the food is lying in the stomach. Not that ptyalin can act in an acid medium—indeed it is destroyed by a degree of acidity as great as that which obtains in the stomach—but the diffusion of the acid into the food is sufficiently slow for the ptyalin to remain unharmed in the middle of the mass for as much as half an hour after it is swallowed. There are two points to note with regard to this action of ptyalin. One is that it stops at the maltose stage, the enzyme having no power of hydrolysis on this

disaccharide. The second is that ptyalin will attack only starch that has been cooked, i.e. in which the grains are broken. This, however, is not the case with the next starch-splitting enzyme we find in the alimentary canal, namely the **amylase** or **amylopsin** of the pancreatic juice. This will act even on unboiled starch and convert it into maltose. But the pancreatic juice and also the intestinal juice contain a further enzyme **maltase** which hydrolyses the maltose to two molecules of glucose so that glucose is the final product of the digestion of the starch we eat. Also in the intestinal juice there is a **lactase** which hydrolyses lactose into glucose and galactose, and also yet another enzyme **invertase** which hydrolyses cane sugar liberating its glucose and fructose. This last enzyme derives its name from the fact that while cane sugar is dextro-rotatory the mixture of glucose and fructose derived by its hydrolysis is lævo-rotatory, the lævulose producing a more powerful left-handed rotation than that produced in the opposite direction by an equal concentration of glucose. The sign of the rotation of a cane sugar solution is therefore inverted on hydrolysis.

It is in the form of these monosaccharides that the fully-digested carbohydrates are absorbed into the blood stream. In this study of these processes of the digestion of carbohydrates we have another illustration of the principle that it is necessary not only to bring the food into a soluble and diffusible form in which it can be absorbed from the alimentary canal and carried to the tissues, but that it is necessary also to bring it into some form which also can be utilised by them. Take the case of cane sugar. It is useless to the tissues. They contain no enzyme that can break it up, and if cane sugar is injected into the blood stream it travels round the

circulation as a foreign body until it becomes excreted by the kidney, and so wasted. Glucose is no more soluble or diffusible than cane sugar, but it is **assimilable**, so that any glucose that enters the blood is, in general, oxidised in the body and does not appear in the urine. It is just as important, therefore, to break down a molecule of a disaccharide into assimilable monosaccharide molecules as to break down the most complex of proteins into its constituent amino-acids.

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CHAPTER XI

THE UTILISATION OF CARBOHYDRATES: THE CHEMICAL MECHANISM OF MUSCULAR CONTRACTION

"On a même cru . . . que rigidité musculaire et réaction acide du muscle étaient deux phénomènes essentiellement liés l'un à l'autre. Il n'en est rien; les deux phénomènes ne sont pas liés l'un à l'autre."—*Claude Bernard*.

WE have just seen that the carbohydrates of the food are broken down to their constituent monosaccharides and are absorbed in this form into the blood stream. Now we must study the uses of these substances in the body. It may be stated at the outset that the chief and most characteristic of these uses is as fuel. This follows at once from the simple observation that during the activity of a muscle its stores of carbohydrates diminish. So that one of the chief subjects with which we shall have to deal in connection with the metabolism of carbohydrates will be the way in which these substances are oxidised—ultimately to carbon dioxide and water—with the liberation of energy, partly in the form of heat and partly as the mechanical energy of movement.

But before we proceed to this main discussion we will point out that in the mammalian body there is abundant provision for the storage of excess carbohydrate material derived from the food, until it is required. In this there is a resemblance to the behaviour of the body towards fat which, as we have seen, is so readily stored in the tissues, and a marked contrast to its behaviour towards the nitrogen of proteins, excess of which cannot be stored under ordinary conditions. On absorption from the intestine the monosaccharides formed by the disruption of more complex carbohydrate molecules are carried in

the portal blood stream to the liver. Here a very considerable amount of storage takes place, the monosaccharide molecules being condensed, under the influence of an enzyme called glycogenase, to form the polysaccharide **glycogen** or animal starch, which is deposited in the liver cells in granules. This glycogen is a white amorphous substance which dissolves in hot water, forming an opalescent colloidal solution. It differs from vegetable starch in giving a *red* colour with iodine and in requiring a greater concentration of a neutral salt—in fact full saturation with ammonium sulphate—to precipitate it. But, like vegetable starch, glycogen is digested by ptyalin to maltose and breaks up entirely into glucose molecules on hydrolysis by acids. It is interesting to note that not only glucose but also fructose and galactose absorbed from the alimentary canal are converted into glycogen in the liver, and that the glycogen so formed seems always to be the same, i.e. it yields only glucose on hydrolysis no matter from what monosaccharide it has been produced. The body has therefore a very remarkable power of converting one monosaccharide into another; it seems to have no difficulty in making glucose from fructose or in giving that twist to the molecule that is necessary for the conversion of galactose to glucose—a process that in the laboratory would not be at all easy to carry out. The reverse twist is given to a glucose molecule taken from the blood when it is converted to galactose to form a component of lactose in the mammary gland. We must remind the reader that glycogen is also formed from many amino-acids (p. 73).

The glycogen thus formed and deposited in the liver is a typical storage product—colloidal and indiffusible.

When the concentration of glucose in the blood tends to fall below its normal value, as for example during starvation or rapid utilisation of sugar, the glycogen is hydrolysed into glucose which then passes in the blood stream to the organs where it is required. This hydrolysis is not merely the result of a disturbance of the physico-chemical equilibrium between glycogen and glucose; it is, as we shall see again later, under the control of the splanchnic nerves. The glucose so liberated passes to the muscles where, after reconversion into glycogen, it plays a fundamental part in the mechanism of muscular contraction. Ordinarily the muscles together contain about as much glycogen as is present in the liver.

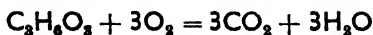
We should just remind the reader that glycogen is not the only reserve material formed from carbohydrates; as we have already mentioned these substances may be easily converted in the body to fat and stored in this form (p. 131).

The great interest in the metabolism of the carbohydrates lies in their utilisation as sources of energy during the contraction of muscles. It is well known that the energy produced by a muscle during contraction is ultimately derived from the combustion of its stored carbohydrates by means of oxygen brought to it by the blood. But the more this question has been studied the more complicated has this process been found to be, and the more unlike the direct combustion of fuel that gives rise to the energy of our non-living engines. For one thing, as has been known for a long time, a muscle will contract on stimulation in an atmosphere devoid of oxygen—in nitrogen gas for example. At first this was explained by supposing that the energy liberated under such conditions was all the same obtained directly

from a combustion process, and that as no oxygen was available to the muscle from external sources, the oxygen actually used for the combustion must have been stored in the muscle as one part of a complex molecule of which other portions were composed of combustible material. It was imagined that when the muscle was stimulated this combustible material was burnt up in the stored intra-molecular oxygen and that the energy for the contraction was thereby obtained. As would be expected on this theory, when a muscle contracts in nitrogen carbon dioxide is *evolved* from it. But it has been known since the time of Helmholtz that a muscle becomes acid when it contracts and the acid produced during the contraction has been identified with dextro- or sarcolactic acid first obtained from muscle tissue by Liebig. Now sufficient of this relatively powerful acid is formed to liberate, from carbonates already existing in the muscle before the contraction, all the carbon dioxide evolved. The act of contraction is therefore not necessarily accompanied by the *production* of carbon dioxide: it therefore does not involve a simultaneous oxidation of fuel.

The more powerfully the muscle is made to contract the more lactic acid is produced in it. When a muscle is heated until it goes into rigor it contracts to its utmost extent, and at the same time liberates its maximum amount of lactic acid. It is evident, therefore, that this formation of lactic acid without oxidation is a very significant step in the complex series of events that constitute a muscle twitch. Direct chemical analysis of muscles before and after contraction has shown that this lactic acid is formed from glycogen, for in proportion as the acid is formed the glycogen content of the muscle becomes diminished. We thus see the significance of the

presence in the muscle of those considerable stores of glycogen to which we have already called attention. With regard to further events, it is found that if the muscle is caused to contract in the absence of oxygen—anaerobically as it is said—the lactic acid once formed in the muscle remains there, and if further contractions are brought about, the further quantities of lactic acid formed are added to that first liberated so that the acid gradually accumulates until the muscle becomes completely fatigued and will no longer respond to stimulation. If now to a muscle in this condition oxygen be admitted, the fatigued condition rapidly disappears—the muscle re-acquires its power of contracting in response to stimulation—while at the same time the lactic acid disappears and there is a considerable formation of carbon dioxide in the muscle accompanied by a very marked evolution of heat. It is evident that at this stage a process of combustion is occurring. It is a matter of direct chemical observation on fatigued muscles placed in oxygen to show that what is burnt up during this process of restitution is a certain fraction—about one-fifth—of the total amount of the lactic acid liberated. It is found that during this combustion process the volume of carbon dioxide produced by the muscle is equal to the volume of oxygen used by it, as would be the case if either lactic acid (or carbohydrate) is being oxidised, thus:—



The remaining larger portion of the lactic acid is apparently in *frog's* muscle re-converted into glycogen, for on exposure of a fatigued frog's muscle to oxygen there is re-formed in it an amount of glycogen equivalent to about four-fifths of

the lactic acid that disappears. But in the muscles of *mammals* no such re-synthesis of glycogen from lactic acid can be observed: in this case the lactic acid diffuses into the blood and is carried to the liver, where its re-conversion to glycogen has been shown to take place. The quantity of glycogen re-formed is, however, at most only about four-fifths of the total amount that breaks down during activity, the other fifth being converted via lactic acid to carbon dioxide and water, so that ultimately glycogen represents the fuel on which the muscle "runs." It should be pointed out that the energy to be obtained by the oxidation to carbon dioxide and water of one molecule of lactic acid is several times as great as that which is liberated during the formation of a molecule of the acid from glycogen, so that the complete oxidation of only a fraction of the total quantity of lactic acid formed during the contraction will suffice to supply the energy that is, of course, needed to reverse the reaction by which it was formed and so to convert the remainder back into glycogen.

From this account it will be realised that the main energy-yielding oxidations in a muscle occur *after* any one contraction and in preparation for the next. The muscle is a kind of machine that in its resting state is "wound up," so to speak, ready to liberate energy without the occurrence of oxidations, so soon as its mechanism is released by a nervous impulse. It is for the rewinding of the machine, for the replacement of its previously liberated energy, that the oxidation of fuel is required. The muscle differs, therefore, very considerably from a steam or petrol engine. For in the engine the oxidation of the fuel and the liberation of the energy go hand in hand. The engine must have oxygen

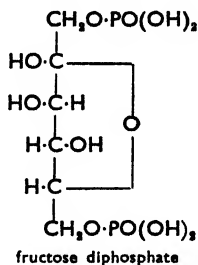
while it is working. On the other hand the muscle requires oxygen only after it has worked—in order to fit it for subsequent activity. The muscle is much more like an electric accumulator charged with chemical energy which by means of a suitable motor can be converted into mechanical work, even in the absence of oxygen, but which has to be replenished after activity by lighting a fire under a steam boiler, i.e. by bringing about a process of oxidation, in order to obtain energy for turning a dynamo and so obtaining the electrical energy necessary for recharging the accumulator. It is not difficult to show that this is the mode of action not only of the isolated sartorius muscle of the frog (from a study of which most of our information on this subject has been obtained), but also of the whole body of a man so far as its muscular activities are concerned. For it is found that during the initial stages of a muscular effort in man the rate at which oxygen is absorbed from the lungs increases only slowly towards its maximum value, so that there is a period at the beginning of the work during which the body is obtaining less oxygen than corresponds to the amount of energy it is producing. The energy is therefore being produced from non-oxidative processes—from the breakdown of glycogen into lactic acid—and the body is getting into "oxygen debt." In other words, unlike our ordinary combustion engines the body has not to wait until it has got the fire going before it can obtain energy—a circumstance that must often have been of considerable help to primitive man in overcoming the forces against which he had to struggle. When the activity is over the oxygen absorption does not return at once to its resting value, but continues for some time at a higher rate until the oxygen

debt incurred at the commencement of the exercise has been paid off. Seeing that this extra oxygen is used for the oxidation of a portion of the lactic acid accumulated in the muscles in order that the remainder can be built back into glycogen it is evident that from the amount of the oxygen debt we can obtain some idea of the total amount of lactic acid that remains in the body at the end of a given effort, and estimating it this way it is found that as much as 100 grms. of the acid may be present at the end of a severe effort on the part of an athlete. One can apply this principle in comparing the athletic capabilities of different individuals by determining the amounts of lactic acid they can stand, i.e. of oxygen debt they can severally incur before collapsing. Seeing that lactic acid does accumulate in this way it is evident that a man can obtain energy by the conversion of glycogen into lactic acid at a more rapid rate than corresponds to the rate at which he can transport oxygen from the air to his active tissues in order to oxidise the acid. In other words, the rate at which he can work during the initial stages of his effort, while he is piling up an oxygen debt, is much greater than that at which he can work when he has reached the stage at which he dare not allow any more lactic acid to accumulate, but must remove it by oxidation as fast as it is being formed. It thus becomes easy to understand why the average speed at which one can sprint 50 yards (which is done practically entirely on "borrowed" oxygen) is much greater than the average speed during a race of a quarter of a mile, in which the efficiency of the runner's respiratory and circulatory oxygen transport is involved. In fact an examination of the world's record times for running various distances shows that the average speed at which the event is run

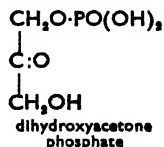
is constant for distances up to 100 yards, and then falls off progressively as the length of the course increases.

Leaving now the consideration of the working of the whole body and returning to the details of the chemical changes that occur during the activity of an isolated muscle, a little reflection will make it evident that the change that we have so far glibly described as the "break-down of glycogen to lactic acid" is far too complicated to take place in a single step, but must involve a whole sequence of stages. It was first discovered that in the alcoholic fermentation of glucose by yeast the initial step consists in the conversion of the glucose into an ester with phosphoric acid; later it was found that such a **hexose phosphate** exists in muscle and constitutes the precursor of lactic acid—hence the

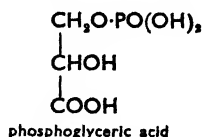
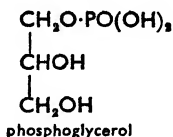
name **lactacidogen**. We now know that this hexose phosphate of muscle is not a single substance but an equilibrium mixture of several such compounds of sugars with phosphoric acid, of which the most significant would seem, interestingly enough, to be the compound of *fructose* with *two* mole-



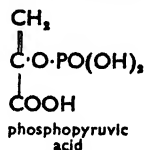
cules of phosphoric acid—**fructose diphosphate**. Evidently then as a first step in its utilisation the glycogen of the muscle is first hydrolysed to glucose and this is transformed to fructose and phosphorylated. The fructose diphosphate molecule then breaks into two halves—each containing a three-carbon chain and one phosphoric acid group. After much investigation the two molecules so formed have been proved to be of the phosphoric ester of **dihydroxyacetone**, which has now actually been isolated from muscle juice. It is



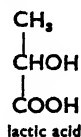
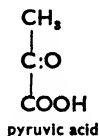
promptly acted upon by the muscle enzyme system, the two molecules reacting by exchange of atoms to form one molecule of the phosphoric ester of glycerol (**glycerophosphoric acid** or **phosphoglycerol**) and one of **phosphoglyceric acid**, the phosphoric ester of glyceric acid, an oxidation product of glycerol. Next, through an intermediate stage involving a shifting of



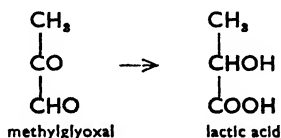
the phosphoric group, this phosphoglyceric acid rearranges itself into **phosphopyruvic acid** (the phosphoric ester of the "enol" form of pyruvic acid) and then at last the phosphoric acid is removed again and **pyruvic acid** remains. From this finally **lactic acid** is formed by reduction with the phosphoglycerol



which we have not yet accounted for, and which, being consequently simultaneously oxidised, gives rise to a single molecule of a triose (three-carbon), phosphate, probably dihydroxyacetone phosphate again. This last reaction may be regarded as a mutual interchange of a :CO group for a :CHOH group between the pyruvic acid and the phosphoglycerol, but of course it does not take place in this naïve manner. The regenerated molecule of dihydroxyacetone phosphate is then caught up in the chain of reactions we have been describing so that it comes about that by a halving of its concentration at each repetition, as it were, it is finally practically all converted into lactic acid.



At this point it will be appropriate to mention that this present-day view of the origin of lactic acid in muscle differs fundamentally from that held until a few years ago when it was supposed that the immediate precursor of the lactic acid was not pyruvic *acid* but pyruvic *aldehyde*, which is the same as **methyl glyoxal**. This idea was based on the occurrence in muscle (in common with most tissues) of an enzyme that rapidly produces lactic acid from this substance. Remembering that glyoxal itself is $\begin{smallmatrix} \text{CHO} \\ | \\ \text{CHO} \end{smallmatrix}$ the relationship of its methyl derivative to lactic acid is at once clearly seen :—



But we now know that muscle juice can convert carbohydrates to lactic acid under conditions in which the glyoxalase enzyme must be inactivated, and what is more when sodium bisulphite, which forms a bisulphite compound with pyruvic acid and so preserves it from further action, is added to the muscle juice, the juice can still produce large quantities of pyruvic acid from carbohydrates but not from lactic acid. Taking all these facts together it becomes evident that the pyruvic acid is the primary product and that the lactic acid is obtained from this by reduction and not the pyruvic from the lactic acid by oxidation. Nevertheless there are some investigators who, finding that under certain conditions muscle tissue can produce lactic acid more rapidly from fructose diphosphate than from an equivalent amount of pyruvic acid, hold the view that the enzyme glyoxalase

is not entirely out of work in the muscle, and that a part, at all events, of the lactic acid is formed from methylglyoxal by its aid.

It is difficult in this brief account to convey an impression of the amount of patient biochemical work of the most brilliant kind that has gone to the establishment of this intricate series of reactions involved in the breakdown of carbohydrate in muscle. But we have by no means related the whole story. As soon as interest had centred on the various phosphorus compounds in muscle and their accurate estimation under various circumstances it was discovered almost simultaneously in England and America that in muscle extracts there exists a substance that slowly breaks down during analytical procedures with the liberation of inorganic phosphate. This **phosphagen** as it was at first called turned out to be a compound of creatine (p. 80) and phosphoric acid—creatine phosphoric acid or **phosphocreatine**. As more of it could be obtained from a fresh muscle than from a fatigued one it looked as if this substance also breaks down during muscular contraction, but this idea was at first not immediately accepted as it upset the energy balance sheet of muscular contraction which had been more or less satisfactorily made out in terms of the breakdown of glycogen (to lactic acid) alone. But that phosphagen does break down during muscular contraction became evident when another remarkable fact came to light. It was found that when a muscle is placed in Ringer's solution containing a small concentration of the sodium salt of iodoacetic acid— $\text{CH}_2\text{I}\cdot\text{COOH}$ —it will still respond, though somewhat feebly, to stimulation, but after it has been made to give about 100 contractions it goes into a permanent condition of rigor and will

respond no more. Strangely enough, however, it is then not white and opaque as a muscle is when it has been sent into rigor by the liberation of an excessive amount of lactic acid, and analysis shows that it does not contain more than a resting concentration of the acid. In other words it has given 100 contractions without forming any lactic acid at all! Evidently a muscle poisoned with iodoacetic acid can obtain energy in some way other than by the formation of lactic acid from glycogen and it is natural to suppose that it is by the decomposition of phosphagen. Phosphagen therefore does break down in the poisoned muscle, and unless we take the somewhat improbable view that the phosphagen is present in the muscle merely in order to enable it to give a hundred twitches should it by remote chance come into contact with iodoacetic acid the phosphagen must break down during the normal contraction of an unpoisoned muscle as well. That this is so has been further proved by taking advantage of the fact that when phosphagen dissolved in a buffer solution is replaced by equivalent amounts of creatine and phosphoric acid alkaline valencies are liberated (simply because sodium phosphate is a somewhat more alkaline substance than sodium creatine phosphate). Now by observing the equilibrium between a normal muscle and carbon dioxide in the atmosphere surrounding it, it has been found that as the result of the first few contractions the muscle comes to take up carbon dioxide, showing that it becomes more alkaline before, owing to the subsequent liberation of lactic acid, it becomes acid and expels the carbon dioxide it had previously taken up. This proves that the breakdown of phosphagen is an early step in the sequence of chemical events occurring during the contraction of a muscle.

But further—it has been known for some time that muscle contains some of its phosphoric acid in that dehydrated form known as *pyrophosphoric acid*, $H_4P_2O_7$, and for still longer that it contains substances of the nature of nucleotides (see Chapter VII). Of these the most significant is **adenylic acid** which is simply a nucleotide composed of the purine base adenine, the pentose (5-carbon) sugar ribose and ordinary or *ortho*-phosphoric acid (H_3PO_4) thus

adenine—ribose—orthophosphoric acid.

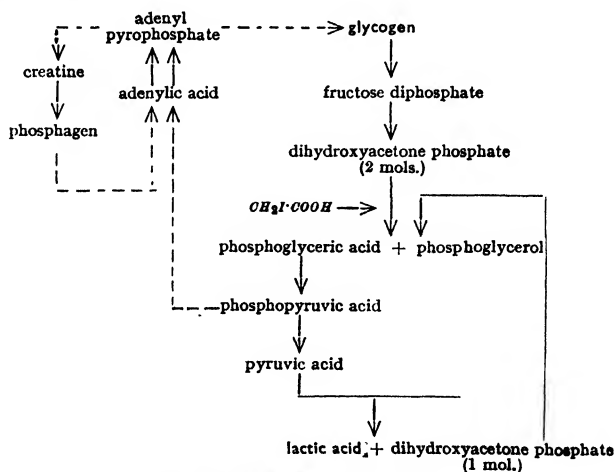
This will be clear if the student has read Chapter VII on the chemistry of the purines and nucleoproteins. It is now known that the adenylic acid and the pyrophosphate of the muscle are united into a remarkable substance **adenyl pyrophosphate** or as it is sometimes called **adenosine triphosphoric acid**. (Adenosine is just the name of the adenine-ribose part of the complex.)

Now this adenylyl pyrophosphate proves to be a substance of prime importance in muscle, for if it be dialysed away the enzyme system remaining in muscle juice becomes quite incapable of converting carbohydrate to lactic acid. Furthermore the muscle enzymes can transfer phosphate to glycogen, forming, as we have seen, hexose phosphates, more rapidly from adenylyl pyrophosphate than from inorganic phosphate so that adenylyl pyrophosphate appears as nothing less than the primary source of the phosphate involved in all the changes we have been describing. Further study of the interlinking of these changes has shown that the adenylic acid thus liberated recoups its phosphoric acid, and so re-forms adenylyl pyrophosphate, at the expense of phosphagen; and that the creatine thus set free has to wait until the

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phosphoric acid is finished with, i.e. until it is liberated from phosphopyruvic acid, before it can recover it. Interestingly enough, in this last-named reaction, adenylyl pyrophosphate also acts as a phosphate carrier between the phosphopyruvic acid and creatine. It is important to realise that all of these reactions take place in this ordered sequence, and that the inorganic phosphate present in muscle does not constitute a pool of phosphate on which all of these substances can draw indiscriminately for phosphorylation.

Thus we find that in the metabolism of muscle there occurs a cyclic transference of phosphoric acid during the course of which it is "borrowed" by the glycogen to form those phosphoric esters that constitute progressive steps in the breakdown of carbohydrate to lactic acid. It may be well if we now summarise all this in schematic form thus :



Carbohydrate breakdown in muscle

The broken line indicates the course of the cyclic transference of phosphoric acid.

Now that we have recounted the main part of this complicated story there are a few further points of interest to add. One is that magnesium ions are in some way necessary as an auxiliary to adenylyl pyrophosphate in order to enable this carbohydrate metabolism to proceed at all. Then again, when the rate of breakdown begins to exceed that of resynthesis so that the adenylic acid is not promptly re-phosphorylated some of it becomes deaminated, its adenine is thus converted into hypoxanthine (p. 115) and there is formed, together with ammonia, a substance known as **inosinic acid** which was isolated from muscle by Liebig almost a century ago: its constitution is:—

hypoxanthine—ribose—orthophosphoric acid

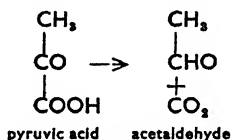
By the removal of adenylyl pyrophosphate in this way a limit would seem to be set on excessive glycogen breakdown and over-activity of the muscle.

An important question that should already have occurred to the reader is "At what point does iodoacetic acid interrupt the course of this carbohydrate breakdown?" Since in muscle juice poisoned with iodoacetate added phosphoglyceric acid is still readily converted into pyruvic, the inhibitory action must be on some earlier stage of the breakdown and is probably exerted on the conversion of the dihydroxyacetone phosphate into phosphoglyceric acid and phosphoglycerol. We have marked this point on our scheme of carbohydrate muscle chemistry, but we should add that iodoacetic acid seems also—at a later stage—to prevent the reduction of pyruvic acid to lactic, although in a muscle poisoned with iodoacetate this effect would not be evident, for in view of the just-mentioned inhibition of carbohydrate metabolism

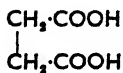
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at an earlier stage no pyruvic acid would be formed at all.

Having thus accounted for the formation of lactic acid in muscle we must now go on to consider the details of its subsequent fate. The greater part of it, which is reconverted to glycogen, apparently retraces the steps of its formation, for the same substances inhibit this resynthesis as prevent the original breakdown: the one-fifth of the lactic acid which is oxidised evidently has to pass through a number of further intermediate steps before appearing finally as carbon dioxide and water. There is little doubt that it is first taken back to pyruvic acid: it used to be supposed that this lost carbon dioxide from its $\cdot\text{COOH}$ group, leaving acetaldehyde:



which on further oxidation would yield acetic acid—a substance that is known to be easily oxidised in the body to carbon dioxide and water. Some support for this view is obtained from the occurrence of traces of acetaldehyde in fresh normal blood. But in the perfusion of muscle with solutions containing pyruvic acid it has been found that succinic and formic acids are produced, but *not acetic acid*. This would seem to indicate that two molecules of pyruvic acid condense to form a six-carbon chain, which splits into a molecule of the four-carbon succinic acid



and two molecules of formic acid, $\text{H}\cdot\text{COOH}$. Now formic

acid is easily oxidised in muscle to carbon dioxide and water, and succinic acid is known, through several intermediate stages involving the loss of a further molecule of carbon dioxide from one of its carboxyl groups, to return to a molecule of pyruvic acid. The net result is that of our two original molecules of pyruvic acid one has been completely oxidised and the other is regenerated to await its turn for oxidation in the same way.

All this in order to bring about the oxidation of a simple sugar molecule! By this time it would not be surprising if the reader is wondering what part all these complicated and interrelated chemical reactions play in the essential process of the development of tension in a muscle fibre, and just which of these decompositions "causes" the muscle to contract. As our knowledge has grown, various suggestions have been made as to the way in which the occurrence of the chemical changes leads to the shortening and pull of the muscle fibres. It is known that a muscle does not change appreciably in volume when it contracts, so that the development of its tension must be brought about by some redistribution of its material. A relatively early theory supposed that this redistribution occurred on account of the breaking down within some cylindrical semi-permeable membrane, situated in the muscle fibre, of large molecules (glycogen) into a larger number of small molecules (lactic acid) with consequent increase of osmotic concentration and an osmosis of water from the outer portions of the muscle fibres into this membrane, and a resulting tendency for the membrane to become more spherical and the muscle fibre to shorten. Another theory supposed that the shortening was due to the swelling of certain colloidal constituents of the muscle

fibre by imbibition of water caused by the liberation of acid, in somewhat the same way as a piece of gelatin will swell if immersed in an acid solution. Yet again it has been supposed that the lactic acid is liberated at some active surface where it produces an increase of surface tension, and so a tendency for this surface, and with it the muscle fibre itself, to contract. But in order to account in this way for the tension developed in a single muscle fibre it would be necessary to postulate that the lactic acid is capable of producing an increase of surface tension many times greater than any that in actual practice has ever been observed. All of these theories were propounded, however, at a time when it was thought that the essential prelude to the contraction was the liberation of lactic acid; but now this lactic acid, once regarded as an essential component of the machine, has been degraded to the ignominious position, as Eggleton says, of "a regrettable by-product." At present, stress is being placed on the circumstance that polarised light shows that the molecules composing the muscle substance are not arranged in a haphazard fashion, but have a regular architecture such as is found in a crystal. The muscle substance is indeed composed of liquid crystals. Attempts are therefore being made to account for the pull of a muscle in terms of the intermolecular forces set up as a result of the re-orientation produced by chemical change in the molecular chains of which its contractile structure is made up, and to apply to this problem the method of X-ray analysis which has been so successful in the elucidation of the molecular structure of solid crystals. But one result of the recent advance in our knowledge of the chemical changes occurring in muscle has been to make it impossible to

ascribe the actual act of contraction to any particular stage of carbohydrate breakdown. It may be that after all we are wrong in trying to do so; perhaps we ought to look at the process the other way round and regard a muscle fibre as essentially a structure possessed of potential energy by virtue of which it is tending all the time to contract of itself. We say "of itself" because when a muscle dies, when the chemical changes within it cease, it contracts: dead muscle is contracted muscle. All this interplay of glycogen, adenylic acid, creatine and phosphates would then be concerned in supplying the energy required for keeping the contractile structure relaxed while it is alive and at rest, and in greater measure still for enabling it to relax again when once it has contracted.

This discussion of the metabolism of carbohydrate in muscle suggests a comparison with the fermentation of glucose to alcohol and carbon dioxide by yeast. But in spite of its interest this is a topic that we cannot enter into fully here. Suffice it to say that by studies on yeast juice it has been shown that here also the sugar is phosphorylated and then via dihydroxyacetone phosphate, phosphoglyceric acid and phosphoglycerol are formed in the initial stages of the fermentation as in muscle. In yeast, similarly, the phosphoglyceric acid further gives rise to pyruvic acid, and here this *does* undergo decarboxylation (under the influence of a decarboxylase enzyme highly characteristic of yeast) with the formation of acetaldehyde and the carbon dioxide that is liberated during the fermentation. The phosphoglycerol is, however, not further attacked by yeast, as it is by muscle: by its hydrolysis it gives rise to the small quantities of glycerol that are an invariable

by-product of alcoholic fermentation; the acetaldehyde is reduced to alcohol not by phosphoglycerol but by the dihydroxyacetone phosphate of an earlier stage of the fermentation, and as in this reaction the dihydroxyacetone phosphate becomes itself oxidised to phosphoglyceric acid (which then forms more pyruvic acid) no further quantity of phosphoglycerol is formed once in the "starting reaction" a sufficient concentration of acetaldehyde has accumulated to oxidise the dihydroxyacetone phosphate completely. But if sodium bisulphite be added to a fermenting mixture the acetaldehyde is trapped, being removed from the reaction by its conversion to its bisulphite compound, so that the yeast is, as it were, kept "running" on its starting reaction and considerable quantities of phosphoglycerol and so of glycerol itself accumulate. The main difference between the metabolism in yeast and in muscle is, then, that in yeast the pyruvic acid is not reduced to lactic acid by the phosphoglycerol but is decarboxylated, by an enzyme the muscle does not possess, to acetaldehyde, and this is reduced by dihydroxyacetone phosphate to alcohol.

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CHAPTER XII

THE PATHOLOGY OF CARBOHYDRATE METABOLISM : GLYCOSURIA : DIABETES

“ If the carbohydrate fires do not burn briskly enough, the fat is incompletely consumed; it smokes, as it were, and the smoke is represented in metabolism by the ketones and derived acids.”—*Macleod*.

It is evidently of very great significance for the study of carbohydrate metabolism that there exists a pathological derangement in which the urine contains considerable quantities of glucose. This condition of **diabetes mellitus** was recognised long ago at a time when the only means of testing for sugar was by the sense of taste, and was in this way distinguished from other derangements (usually of water balance) in which there was observed a passing through (*διαβαίνειν*) the kidney of abnormally large quantities of tasteless, i.e. sugar-free, urine (**diabetes insipidus**). Naturally the first step in the investigation of this condition of sugary diabetes is the study of the various circumstances under which sugar may appear in the urine. For this purpose the behaviour of the kidney towards the blood-sugar must first be clearly realised. Blood contains normally about 0·1 per cent. of sugar, which is all glucose. According to modern theory this glucose filters through the kidney glomeruli in unchanged concentration and then is reabsorbed with remarkable completeness by the tubules. The kidney can keep back in this way all the glucose provided its concentration in the blood does not exceed 0·18 per cent., i.e. nearly double the usual amount ;

above this **threshold** concentration, however, the tubules cannot reabsorb the whole of the sugar so that some is left over in the urine and the condition of **glycosuria** supervenes. The behaviour of the kidney towards glucose is therefore very different from its behaviour towards the **non-threshold** substance urea, which appears in the urine, no matter how small its concentration in the blood. From these considerations it will be evident that one possible cause of glycosuria will be the presence of an excessive concentration of glucose in the blood. But so efficient in the normal body are the means for storing excess glucose as glycogen that it is not at all easy to exceed the threshold blood-sugar concentration even by eating glucose itself, and it is impossible, owing to the more gradual digestion and absorption, with starch. The production of such an **alimentary glycosuria** requires the consumption of something like a pound of sugar. In investigating the carbohydrate metabolism of a patient it is usual to perform a glucose tolerance test by administering a dose of 50 grms. of glucose by the mouth; in a normal subject this never sends the blood-sugar concentration above the renal threshold value. On the other hand it is possible for glycosuria to arise even with a normal concentration of sugar in the blood if the power of the kidney tubules to reabsorb the sugar from the glomerular filtrate is in some way impaired. There then arises a **renal type of glycosuria** which is not indicative of any serious *metabolic* disturbance; when it occurs in man it is referred to as **diabetes innocens**. The condition can be imitated in animals by the injection of the drug **phlorizin** obtained from cherry and apple tree bark; this renders the kidney permeable to sugar, and so is

useful when one wishes to deplete the carbohydrate stores in an experimental animal or to determine whether some particular substance gives rise to glucose during the course of its metabolism. Another method of producing glycosuria was discovered by Claude Bernard during an attempt to study the effect on the liver of stimulating the nucleus of the vagus nerve. He was surprised to find that mere puncture of the floor of the fourth ventricle of the brain, even in the absence of any electrical stimulation, led to a rapid turning out of glucose from the liver, with a consequent increase in the blood-sugar concentration (**hyperglycæmia**) and the appearance of sugar in the urine. When the physiology of this effect was studied in more detail it was found to be due not to any effect via the vagi, but to impulses passing down the spinal cord and out by the splanchnic nerves to the liver, assisted by adrenaline secreted by the suprarenals—which must be intact for the effect to be obtained at all. As this **puncture diabetes** can be obtained in animals whose livers have been freed from glycogen by starvation it cannot be entirely due to a mere mobilisation of previously stored carbohydrate reserves, but must involve the formation of new sugar (**gluconeogenesis**) from non-carbohydrate sources such as amino-acids. A similar effect can be produced by a large injection of adrenaline itself.

Lastly, the most fundamental discovery of all in relation to diabetes mellitus was made accidentally by von Mering and Minkowski in 1889. Working in Strassburg (which was then in Germany) they succeeded in removing the whole of the pancreas from a dog with the object of observing the effect on the digestion of fat, and were surprised to find that as a consequence the animal

developed a severe glycosuria, together with the other serious symptoms associated with sugar diabetes. That this was not the result of the absence of the pancreatic juice from the intestine is shown by the fact that mere ligation of the pancreatic duct does not produce these symptoms, nor do they develop if the pancreas be removed from its normal position and sewn up into the subcutaneous connective tissue, provided that to the grafted organ a good blood supply is maintained. It is evident, therefore, that it is some **internal secretion** or **hormone**, produced by the pancreas, which is responsible for keeping the carbohydrate metabolism normal. This is also shown by the observation that in a depancreatized pregnant bitch the internal secretion of the pancreases of the embryos diffusing across the placenta into the maternal blood is sufficient to protect the mother from diabetes until the pups are born and her supply of the hormone consequently fails. The student who is familiar with the histology of the pancreas will remember that it is composed of two kinds of tissue. There are the ordinary glandular alveoli lined by the cells which produce the pancreatic juice — the external secretion — and also a certain amount of tissue consisting of masses of cells not arranged in alveoli, which cell masses are named after their discoverer **islets of Langerhans**. These islets have a particularly good blood supply, and therefore are well situated with regard to the formation of an internal secretion. It has been shown conclusively that these islet cells are quite distinct in origin and function from the cells that form the external secretion. The internal secretion produced by the islets of Langerhans, and passing from them into the blood, is essential for the normal utilisation of glucose in the tissues. If that internal secretion be not present glucose

accumulates in the blood and so gets over into the urine. Meanwhile the stored glycogen of the body is drawn upon — the tissues, as it were, call for it — but this is of no avail, for the glucose coming from the glycogen can no more be utilised than that which comes from the alimentary canal. At the same time the tissue proteins break down at an abnormal rate, and there is an excessive gluconeogenesis from the amino-acids to which they give rise. We know this from the fact that the ratio of the amounts of glucose and total nitrogen in the urine settles down to a constant value, which indicates that both the sugar and the nitrogen are being obtained from the same source, namely the tissue proteins. In a phlorizinised animal in which *all* the sugar formed leaks through the kidney this $\frac{D}{N}$ (dextrose to nitrogen) ratio settles down to a value of about 3.65, and the more nearly this value is approached in other cases of patients or experimental animals the more serious is the disturbance of the utilisation of sugar in the body. Diabetes mellitus in man possesses most of the characteristics of this **pancreatic diabetes**, although its occurrence is not invariably associated with visible pathological changes in the pancreas itself. The large concentration of sugar in the glomerular filtrate hinders the reabsorption of water by the kidney tubules—hence the “diabetes” of excessive quantities of urine and the consequent continuous thirst. The excessive breakdown of tissue proteins is the cause of the emaciation usual in a diabetic patient, and the loss of this protein is naturally a more serious feature of the disease than the mere wastage of the sugar itself. But by far the most serious symptoms of the disease result from the circumstance that when the metabolism of carbohydrates is to this extent deranged

the oxidation of fats is also interfered with and becomes incomplete. Instead of being burnt right up to carbon dioxide and water, the fatty acids remain in a half-oxidised condition in the form of **aceto-acetic acid**, $\text{CH}_3\cdot\text{CO}\cdot\text{CH}_2\cdot\text{COOH}$, and **β -hydroxybutyric acid**, $\text{CH}_3\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{COOH}$. The formula of acetoacetic acid will be readily understood when it is realised that it expresses the replacement of one of the hydrogen atoms of the methyl group in acetic acid by the acetyl group $\text{CH}_3\cdot\text{CO}\cdot$. The formula for β -hydroxybutyric acid is at once obvious. From aceto-acetic acid **acetone** $\text{CH}_3\cdot\text{CO}\cdot\text{CH}_3$ is readily formed by the loss of CO_2 from the carboxyl group — hence these substances are usually referred to together as the “acetone bodies.” The acetone can be smelt in the breath of a diabetic patient; together with the other acetone bodies it is excreted in the urine where it can be tested for by taking advantage of the fact that if the urine be first saturated with ammonium sulphate to remove interfering substances and made alkaline with a little ammonia, a drop or two of sodium nitroprusside solution will then give a deep purple “permanganate” coloration with any acetone or acetoacetic acid that may be present (Rothera’s test). The rate of development and final intensity of this colour depend on the concentration of acetone bodies present, and so may be used as a rough measure of the amount of these substances being excreted. These substances are poisonous, firstly, because they are strong acids, and so disturb the neutrality regulation in the tissues. Evidence of this disturbance is shown by the increased ammonia contained in the urine. Whenever abnormal amounts of acid appear in the blood, some of the ammonia, which otherwise would be converted

to urea in the liver, is kept over to neutralise the harmful acid. Further, the tension of carbon dioxide in the alveolar air is reduced, because the hydrogen ion concentration of the blood becomes increased, so that there is more powerful stimulation of the respiratory centre, and, therefore, increased ventilation of the lungs, with a consequent more complete washing out of the carbon dioxide. This reduction of the carbon dioxide content of the alveolar air—and so of the carbonic acid content of the arterial blood—constitutes a further means whereby the increased acidity due to the foreign acids may be partially compensated. But it is not only by virtue of their acid properties that these acetone bodies are poisonous, for their neutral sodium salts also exert a harmful action on the body. If the diabetic condition is not treated they accumulate in the blood and eventually poison the nervous system, bringing on a condition of coma which is almost invariably fatal.

We have said that the acetone bodies are produced from fats whenever carbohydrate metabolism is interfered with; it is not surprising, therefore, to find them produced not only during the metabolic disturbance associated with sugar diabetes, but also whenever the tissues are robbed of carbohydrates as, for example, when these substances are withheld from the food, or during a period of complete starvation, or again during the inanition that results from the excessive vomiting that accompanies so many clinical disorders. A normal person can easily produce such a **ketosis** in himself by abstaining from sugar, bread and potatoes for a couple of days, and restricting his diet to fat meat, butter, eggs, sardines and similar foods containing excess of fat. The urine will then be found to give a positive Rothera's test which, however, will promptly

disappear so soon as a diet containing a normal proportion of carbohydrates is resumed.

Before we leave our consideration of these acetone bodies we will remind the reader that Embden showed that they are formed on perfusion through the liver of fatty acids containing an even number of carbon atoms, i.e. of the fatty acids occurring in natural fats (p. 138)—in fact we may say that they represent the residue left when a long carbon chain originally containing an even number of carbon atoms has had its links removed two by two until only two pairs of carbon atoms remain. The normal organism can oxidise these residual four-carbon chains completely to carbon dioxide and water, but the diabetic makes only an incomplete attempt at the β -oxidation of these substances—an attempt that is manifested by the presence of the :CO group in the β -position in acetoacetic and of the :CHOH as the β -group of β -hydroxybutyric acid; apparently it is in the completion of this final β -oxidation that the simultaneous utilisation of carbohydrates is essential. Since these acetone bodies are not produced from acids containing an odd number of carbon atoms (they yield glucose) it has been suggested that ketosis in a diabetic might be prevented by replacing most of the fat of his diet by the synthetic glyceride of the 17-carbon margaric acid, but, unfortunately, there is some doubt as to whether this substance is at all well absorbed from the intestine. Lastly, in connection with acetoacetic acid, it must be mentioned that this is formed not only from the even-carbon higher fatty acids in the way we have described, but, remarkably enough, is produced when acetic acid is perfused through the liver, and also from certain of the amino-acids such as phenylalanine, tyrosine and leucine.

We must now return to the consideration of the disease diabetes. It is generally known that until comparatively recently the only available treatment for this disease consisted in giving a considerable amount of sodium bicarbonate to neutralise the acids, and in keeping the patient thin by starvation so that he possessed a smaller amount of tissues between which to share his diminished supplies of pancreatic hormone. It was found to be possible in many cases slowly to increase the tolerance for carbohydrates by giving gradually increasing quantities, and ensuring that the tolerance limit at any time was never exceeded. But now at last it has become possible to treat the disease in a more fundamental fashion by supplying to the patient an extract of pancreas containing the active hormone produced by the islets of Langerhans. The existence of this hormone has long been recognised, and the name **insulin** by which it is known is far from new, but it was not until 1922 that Banting and Best, working in Toronto, brought to a successful issue the long series of attempts at its extraction. This seems to be due to the circumstance that the earlier workers failed to realise that the proteolytic enzyme trypsin formed in the pancreatic alveoli has a powerful destructive action on the hormone. As soon as there were devised methods of extraction that did not involve the exposure of the insulin to the danger of digestion by trypsin, potent extracts were obtained. The first method proposed was that of extracting the pancreas of an animal whose pancreatic duct had been ligatured some time previously, and whose enzyme-forming cells had in consequence become completely degenerated, leaving the islets of Langerhans intact. Another suggested method was the extraction of foetal pancreas in which only the islet tissue is developed and the

enzyme-secreting alveoli are not yet functional. But these methods require such specialised material as a starting point that they are not suitable for the production of insulin on a large scale. The commercial process in use at present consists in the extraction of the whole adult pancreas, which is readily obtained from the slaughter-house, with alcohol of sufficient strength to precipitate and so render inactive the trypsin that is present. The insulin is not affected by the alcohol, and can be further concentrated into a suitable medium. The extract so prepared produces an amazingly beneficial effect on a diabetic patient. Under its influence the sugar disappears from the urine, the production of the poisonous acetoacetic and β -hydroxybutyric acids is stopped and the glucose concentration of the blood returns to a normal value. When insulin is injected into a healthy non-diabetic animal the blood-sugar concentration is also reduced—in this case far below the normal value—and by the time it has sunk to 0.07 per cent.—approximately half the normal—the nervous system suffers so much from deprivation of sugar that the animal goes into convulsions and ultimately dies. This observation is of extreme importance, for it indicates that an excessive dose of the hormone may produce very undesirable results; but at the same time it also furnishes us with a method of standardising insulin preparations. The relative amount of the active principle contained in a given preparation is determined by observing the quantity of the preparation required to reduce the blood-sugar concentration of rabbits or mice to the convulsive level in a given time. The amount of insulin required to bring about this result in three hours after injection into a fasting rabbit weighing two kilos. is defined as the physiological unit of the

substance ; for convenience, however, the unit by which insulin is sold and administered clinically is one-third of this amount.

The danger attending the administration of a dose of insulin in excess of the physiological requirements of the patient would constitute a serious drawback to the use of this material were it not that the untoward symptoms can be easily recognised before the dangerous convulsive stage is reached, and can be just as easily counteracted by the administration of a dose of glucose by the mouth or, in urgent cases, by intravenous injection ; a more serious drawback is presented by the circumstance that insulin is too unstable to resist the digestive action of the ferments of the alimentary canal. Therefore it cannot be taken by the mouth, but has to be administered in the form of repeated subcutaneous injections. In this respect insulin presents a marked contrast to the thyroid hormone, which is readily absorbed unchanged when given with the food.

It is only natural that the mode of action of a substance of such great practical and theoretical interest as insulin should have been the object of very detailed study. Seeing that the administration of insulin will permit a diabetic patient to live a more or less active life instead of dying a lingering death in a hospital ward, it is evident that the more we know about the exact rôle of insulin in metabolism the more we shall understand of the derangement that has taken place in the patient. We have already mentioned that insulin causes a marked fall of blood-sugar concentration from the normal value both in non-diabetic animals and in normal human subjects. But the conditions obtaining in the intact animal are too complicated to allow us to determine from such observations the primary effect of insulin. If, however, we

use an animal whose central nervous system has been destroyed, whose viscera have been removed, and whose liver has been tied off from the circulation—if we use, that is, a carcass composed of practically only the skeleton and muscles—and perfuse it with a suitable Ringer's solution containing the same concentration of glucose as exists normally in the blood (so that the glucose concentration in the circulating fluid is maintained constant and is not allowed to fall as it would in the intact animal), then on adding insulin to the perfusion fluid it is found that, judging by the amount of oxygen absorbed and carbon dioxide produced in a given time, the rate at which carbohydrate is being burned in the carcass is somewhat increased, while at the same time a very considerable amount of glycogen is deposited in the muscles. Further, all the sugar that disappears from the perfusing fluid under the influence of insulin can be accounted for by the sum of what is burned and that which is deposited as glycogen. Evidently the primary effect of insulin is to encourage the combustion of carbohydrate and the deposition of glycogen. When, however, insulin is injected into an intact animal (which differs from the perfused carcass just considered by having no continuous supply of sugar from outside) the conversion of the blood-sugar into muscle glycogen leads to a secondary reaction whereby nervous impulses pass to the liver and cause the hydrolysis of its glycogen in an attempt, as it were, to maintain the normal concentration of glucose in the blood. But if the dose of insulin is sufficiently large the amount of glycogen in the liver will not suffice to maintain the normal level of blood-sugar and ultimately the blood with subnormal sugar content will constitute an abnormal environment, and as a result of depletion of sugar the central nervous system will

behave abnormally and send out to the muscle those uncoordinated impulses that give rise to the hypoglycæmic convulsions previously mentioned. In these convulsions a very considerable amount of carbohydrate is burned, so that in the end, as a result of the insulin, the animal will come to possess very little glycogen in its liver and muscles and very little sugar in its blood. In fact the glycogen content of the muscles may be completely exhausted by the occurrence of the convulsions at the time when there is no glucose in the blood for replenishment. It is found that when this happens the muscle goes into an alkaline rigor which is evidently similar to that which is observed in a muscle whose glycogen is put out of function by means of iodoacetic acid (p. 195). Now, during voluntary muscular exercise there is also a considerable usage of the carbohydrate reserves of the body, but yet the blood-sugar does not become markedly reduced in quantity during muscular work nor do the muscles become so exhausted of glycogen as to go into rigor. The reason is evidently that when the pre-formed glycogen has been used up, further supplies of this substance are produced from other materials, notably from proteins by way of amino-acids in the liver. This method of glycogen formation is one with which we are already familiar (p. 73); seeing that it does not take place in an animal under the influence of an excessive dose of insulin it would seem that this hormone not only facilitates the deposition and utilisation of glycogen, but also regulates, in the sense of retarding, the formation of glycogen from amino-acids and proteins. And this accords with what we observe in the cases of diabetics who are not receiving a sufficient supply of insulin, for, as we have mentioned, their tissue proteins break down at an abnormally great

rate and give rise to a considerable proportion of the sugar that is excreted. When the amount of insulin is normal the formation of sugar from non-carbohydrate sources is neither completely inhibited nor is it excessive ; it occurs at a rate appropriate for the maintenance of the balance of the metabolism of the whole organism.

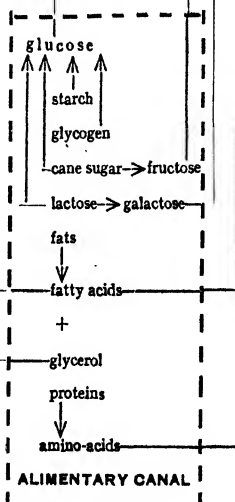
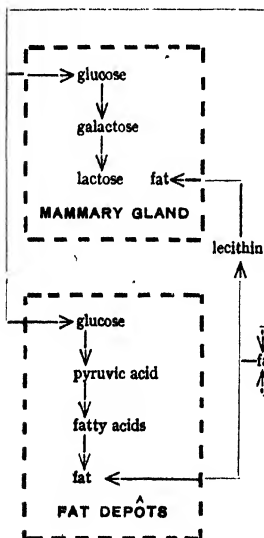
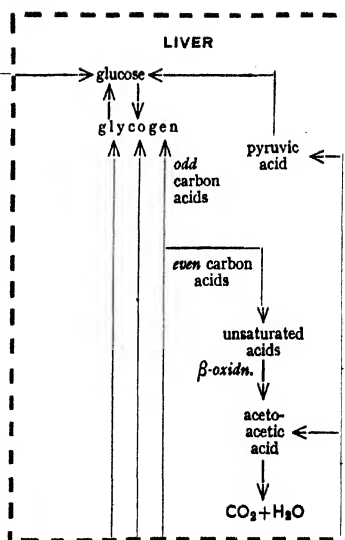
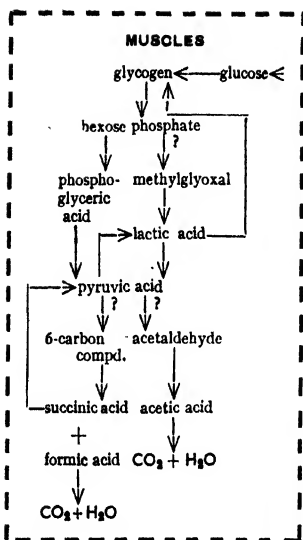
Insulin has been obtained in the crystalline form, and it turns out to be a protein combined with an active group. The protein portion contains sulphur that it readily gives up to caustic alkali, but this sulphur seems not to belong to the active portion of the molecule. This active group itself seems to be too unstable to exist for long in the free condition, but that it has at least a fleeting existence is shown by the circumstance that solutions of insulin have been prepared whose activity is greater than corresponds to the amount of the crystalline substance present. In view of this chemical constitution it is not surprising that insulin should be destroyed during the earlier attempts at its preparation and when administered by the mouth.

Thus it will be seen that, thanks to the skill that has gone to the isolation of insulin and no less to the elaboration of accurate methods for following the changes in blood-sugar concentration produced under its influence, our control over the disease diabetes has been immeasurably extended. But, nevertheless, it must be admitted that our knowledge of its causation is not yet complete. For insulin does not *cure* diabetes ; it must be administered in the same regular doses as long as the patient lives. And that this is not always merely the supplying from outside sources of a hormone that the patient is failing to produce for himself is shown by the surprising fact that human diabetic patients have been found whose pancreases have yielded, post mortem, as much insulin as those of normal

individuals. Diabetes mellitus in man is therefore not necessarily due to an absence of insulin as it is in a depancreatized dog. One is tempted to think that in certain cases the action of insulin may be in some way depressed by the simultaneous presence of some antagonistic substance, and this is borne out by the now well established fact that a dog will remain in a much more nearly normal metabolic condition, and survive for months instead of weeks, after depancreatization if it be deprived of the anterior lobe of its pituitary as well. It is better off without two glands than without only one! The explanation of this remarkable result has been shown to be that the anterior lobe of the pituitary secretes a **diabetogenic hormone** which works antagonistically to insulin, and whose excessive production may be the essential abnormality in those "insulin resistant" cases of diabetes where the administration of the pancreatic hormone is without its usual beneficial effect.

SUMMARY

This brings us to the end of our study of the details of the chemical changes suffered by fats and carbohydrates in the body. We will therefore summarise in the following diagram the chief metabolic paths that we have described in the last four chapters:—



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CHAPTER XIII

THE HUMAN MACHINE: ITS FUEL REQUIREMENTS AND ENERGY OUTPUT

“ Nous sommes créés pour transformer ce que nous absorbons des choses de la terre, en une énergie particulière et d'une qualité unique.”—*Maeterlinck*.

WE have dealt with the details of the chemical stages involved in the utilisation as fuels of each of the three classes of foodstuffs, but we have yet to consider the body as a whole with regard to its total fuel requirements. This is a natural and important question with regard to the working of any machine. In order to answer it we must first know the total amount of energy that the machine expends in the course of a day (including any heat loss that may occur), and also the amount of energy to be obtained from a given quantity of fuel under the conditions of working of the machine. Now energy is required by the body for the performance of muscular movements and for the maintenance of its temperature. As a matter of fact practically the whole of the energy which we expend as muscular work during our ordinary everyday activities is converted ultimately into heat. For example, the energy expended in walking or cycling along a level road is all used in overcoming frictional resistances: that exhibited by a manual worker such as a blacksmith goes to heating the material he is shaping by his vigorous blows: even the energy displayed by the muscles of my hand as I write these words is being lost as heat owing to the friction between my pen and the page over which it moves. It is only when we use our muscles

for storing up energy, say, by lifting a weight through a vertical distance, that work is done that does not immediately appear as heat. It follows from these considerations that if we could measure the rate at which heat is produced by a living creature we should know the rate at which he is expending energy, and hence know also the total quantity of combustible food he will require for use as a source of this energy. Amounts of heat energy are expressed in calories, a calorie being the amount of heat required to raise the temperature of unit mass of water one degree centigrade. For physical purposes it is often convenient to choose 1 grm. as the unit mass of water, but in the study of the energy exchanges of the body it is more convenient to take a unit a thousand times as large. This is called the **large Calorie** (spelt with a capital "C")—it is the amount of heat required to raise the temperature of a kilogram of water by 1° C. It is simplest for our purposes always to express all quantities of energy in these heat units. We can do this because it has been shown by very careful experiments that a given amount of mechanical work always corresponds to a proportionate amount of heat energy. It is the ratio of the amount of mechanical work to the amount of heat to which it corresponds that is termed the mechanical equivalent of heat. In physical experiments it is usual to measure the amount of heat given off by a body, during any change, in some form of calorimeter—a vessel in which the heat is measured by the rise of temperature of a known mass of water. And usually it happens that the changes investigated in such experiments are so rapid that it is justifiable to assume that no appreciable amount of cooling takes place during the experiment, so that the whole of the heat is retained

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by the calorimeter and its contents. This is the principle, for example, of the **bomb calorimeter** for the estimation of the total energy of combustion of such materials as foodstuffs. A weighed amount of the material is placed in a stout airtight steel bomb filled with compressed oxygen; it is ignited electrically; and the heat generated during the rapid combustion produces a rise of the temperature (which is noted) of a known mass of water in which the bomb is immersed. From these data the amount of heat liberated can at once be calculated, allowance being made of course for that portion of it that goes to raising the temperature of the bomb and other metal parts of the calorimeter itself. From an animal, however, the heat is given off slowly and continuously, and the time required for the observation is usually so long that unless the amount of heat lost by cooling from the calorimeter were taken into account a very erroneous estimate of the total amount of energy expended by the organism would be obtained. There are several ways in which this difficulty has been overcome in practice. Rubner avoided it in experiments with animals by noting the rise of temperature occurring in his calorimeter in a given time, and then substituting for the animal under test a gas flame whose size was adjusted until it produced the same rate of rise of temperature as did the animal previously. He then noted the rate at which the gas was being burnt; and knowing the amount of heat liberated during the combustion of unit volume of the gas, he could calculate the rate at which the animal was producing heat, and so estimate the rate at which its oxidative metabolism was proceeding. For small animals and isolated muscles A. V. Hill has found that ordinary thermos vacuum flasks make good calorimeter vessels. The rate

of loss of heat from these is very small, and in any case can be determined under any given experimental conditions and allowed for. But the heat insulation of a calorimeter chamber large enough to contain a man is a problem of a totally different order of difficulty from that of producing a vessel suitable for the measurement of the heat evolved by a small animal or an isolated organ. Atwater and Benedict, working in America, have, however, succeeded in constructing such a chamber (see Fig. 10). They do not attempt to prevent loss of heat from the room containing the subject by means of insulating material, but by enclosing it in a larger chamber the inner walls of which are kept adjusted, by means of electric heaters, to exactly the same temperature as that of the outer walls of the inner chamber where the subject is. This temperature control is brought about by means of thermo-electric junctions. It is evident that, since a body loses heat only as a result of differences of temperature between itself and its surroundings, if the chamber and its surroundings are maintained at exactly the same temperatures it will lose no heat. Or we can say that the electric heaters in the outer chamber of the Atwater-Benedict calorimeter replace the heat of the inner chamber just as fast as it is lost. The heat produced by the subject is not allowed to accumulate in the inner chamber, and so to raise its temperature: it is carried away and measured by what is called the method of "continuous flow." That is, a current of water is circulated through a system of pipes in the chamber, and is warmed by the heat evolved by the man inside. The rate at which the water flows is measured, together with the respective temperatures at which it enters and leaves the chamber. We thus know that in a noted time a certain mass of water

has had its temperature raised by a certain number of centigrade degrees; we can easily calculate how many kilos. of water would have had its temperature raised by

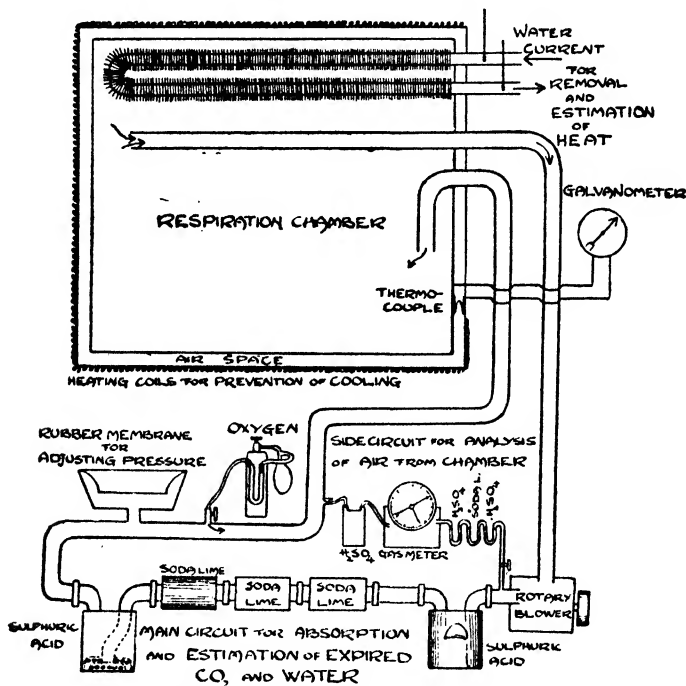


FIG. 10. A diagram of the Atwater-Benedict respiration calorimeter. (Drawn from the figures and descriptions of Benedict and Milner.)

one degree by this amount of heat, which is the same thing as the number of Calories of heat produced by the subject in the given time. Provision has of course to be made for adding oxygen to the chamber at the rate at which the subject uses it, and for absorbing the carbon

dioxide and water vapour he produces. The oxygen is added from a cylinder, and the amount of it used during any experimental period is measured by weighing the cylinder on a specially strong but sensitive balance before and after use. The carbon dioxide and water are absorbed and estimated by pumping the air from the chamber continuously through weighed absorbers containing strong sulphuric acid and soda-lime respectively. As each experiment lasts for several days the chamber is furnished with table, chair and bed (and, being in America, with a telephone), and is provided with air locks for the admission of food and the passing out of excreta. The amounts of energy obtainable from the combustion of each article of the diet, and also that remaining in the constituents left unabsorbed in the fæces or excreted as waste in the urine, were measured by burning known amounts of these materials (dried where necessary) in the bomb calorimeter. Allowance is made for such items as the heat taken up in evaporating the water that leaves the chamber in the form of vapour, the material excreted by the subject in sweat (this latter being estimated by analysing the wash water from his clothing!), any slight difference in composition of the air in the chamber at the beginning and end of the experiment, and any gain or loss of actual body substance by the subject. In this way it is possible to draw up a very exact balance sheet of the amounts of energy entering and leaving the chamber during an experimental period, and when this is done it is found that the more carefully the measurements are made the more closely do these two amounts of energy agree. In one of Benedict's best series of experiments the difference amounted to only 0.1 per cent. of the total amount of energy involved — a truly remarkable agreement when one

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considers that in this series of experiments the energy output was measured over periods amounting in the aggregate to almost six months. In some of these experiments the subject was continuously in a state of physical rest; in others he performed work on a stationary bicycle placed in the chamber for the purpose: but in each case the result was the same. No matter whether he performed as little or as much work as possible the amount of energy liberated in the body of the subject by the combustion of a given amount of food was, as nearly as could be measured, the same as the amount of energy that would have been liberated by the combustion of the same amount of the same foodstuffs in the bomb calorimeter. A living organism is therefore not in any privileged position with regard to energy supply: it can extract no more energy from its food than that which would be liberated when that same food is burned in a non-living machine. Incidentally the same experiment shows us that if energy is not created, neither is it destroyed by living matter, so that the same total amount of heat is inevitably formed by the oxidation of a gram of fat, carbohydrate or protein, in the human body as would be developed by the combustion of this amount of these materials outside it. In other words, none of the chemical energy is wasted during its conversion in the body into mechanical energy, and from this ultimately to heat. It is conserved just as completely as during the energy transformations in non-living matter; the law of the **conservation of energy**, therefore, applies to the living animal no less than to the dead earth on which it lives. Indeed, the law of conservation of energy was first deduced from the observation that the venous blood of the inhabitants of hot climates was of a brighter red colour, and therefore richer in oxygen than that of

dwellers in colder regions. This means that less oxygen is used from the arterial blood in the case of the tropical native, because he requires less oxidation in his tissues since he loses less heat to his surroundings, and so has less to replace than his more chilly neighbour. This suggested the idea that a given amount of oxidation must inevitably give rise to the same amount of energy, and that, therefore, the only method of controlling the heat production was the regulation of the amount of oxidation occurring.

The organism, then, for all its warm throbbing life—despite, it may be, its keen mentality, developed through long ages of painful evolutionary experience—has no power to create the minutest fraction of that energy which it requires for its continued existence; neither is it able to destroy even the weakest of the forces which influence it in its environment, no matter how much these may hamper its activities; its behaviour is always circumscribed by the same inevitable laws that operate in those changes where life is not concerned.

Now these considerations are of the utmost importance, for they form the foundation of the science of dietetics. For on the one hand they enable us to say how much energy will be yielded to the body by the combustion of a given amount of any foodstuff—it will be just the same as the energy yielded by the combustion of the foodstuff (with the same degree of completeness) in the bomb calorimeter, or, to put it in a form more useful for practical purposes, it will be the total energy obtainable by the combustion of the food minus the energy still remaining in the waste products formed in the body from that food. On the other hand, these results also justify us in calculating the amount of energy being expended during a given

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time in the body from the amounts of the various materials being burned in the body in that time, and these can in turn be deduced from the amounts of the products of combustion, i.e. the amount of carbon dioxide, water and urea produced.

With regard to the first of these we will simply mention that one of the most instructive experiments that the student can perform on this matter is to determine his own total Calorie intake for a day. All that is necessary is that he should provide himself with a suitable balance—most conveniently one in which the readings are given directly by a pointer on a scale without the use of weights. During the day of the investigation all the different articles of diet should be served to him on separate saucers, which, with their contents, are to be weighed before and after he has eaten as much as he desires from each. In this way he determines the total weight of each foodstuff eaten during the day. The table on pages 233-234 will then give him the average composition of most of the foods he is likely to be interested in. These data refer to the edible portion of the various foods, the percentages of water, salts and indigestible waste not being recorded. With their aid it will be easy to compute the total daily consumption of proteins, fats and carbohydrates, and then by using the results of bomb calorimeter determinations which show that the oxidation of

	1	gram. of protein	yields	4.3	Calories,
	1	gram. of carbohydrate	"	4.2	"
and	1	gram. of fat	"	9.3	"

to add up the total number of Calories of energy taken into the body per day. Of course the heats of combustion of all proteins are not quite the same, nor are those of all carbohydrates or of all fats, but for practical purposes

COMPOSITION OF TYPICAL FOODS*

This table gives the percentage of proteins, fats and carbohydrates in the *edible portion* of each foodstuff: the residue, consisting of water, mineral salts, and indigestible material, is not recorded.

MEATS						
			Protein	Fat	Carbo-	hydrate
			%	%	%	
Bacon, back, fat	1.9	87.7	—	
lean	19.3	11.6	—	
Beef, rump steak, lean	21.9	7.3	—	
middle rib	20.0	16.5	—	
kidney	18.1	2.6	—	
liver..	19.9	3.2	4.4	
suet	1.2	93.3	—	
Chicken, breast	24.6	0.2	—	
Ham, lean	20.3	12.3	—	
Lamb, shoulder, lean	18.7	8.7	—	
Mutton, leg, lean	21.1	7.0	—	
Pork, loin, lean	21.4	11.1	—	
Veal, hindquarter	20.4	2.7	—	
FISH						
Cod..	17.5	0.1	—	
Haddock, smoked	19.6	0.3	—	
Herring, fresh, male	18.6	10.9	—	
bloater, male	17.8	14.9	—	
kipper	18.8	14.9	—	
Mackerel	19.1	10.7	—	
Plaice	15.7	2.0	—	
Salmon	18.6	15.8	—	
Skate	24.2	0.1	—	
Sole	18.8	1.8	—	
Whiting	17.7	0.2	—	
DAIRY PRODUCTS						
Butter	—	81.6	—	
Cheese, cheddar	25.2	33.4	—	
Eggs, without shell	12.3	11.26	—	
Lard	—	100.0	—	
Margarine, average	0.2	84.8	—	
Milk	3.3	3.6	4.8	

* These data are quoted from *Analyses and Energy Values of Foods*, by kind permission of Professor R. H. A. Plimmer and H.M. Stationery Office.

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VEGETABLES

			Protein	Fat	Carbo- hydrate
			%	%	%
Artichokes, Jerusalem	1.9	0.03	17.4
Beans, broad, boiled	6.1	0.2	14.1
French	1.9	0.1	4.8
scarlet runner	1.3	0.1	3.9
Beetroot	1.2	0.1	6.2
Cabbage	1.45	0.1	6.3
Carrots	1.2	0.1	9.6
Cauliflower	1.9	0.2	5.9
Lettuce	1.1	0.2	1.9
Marrow, green, boiled	0.4	0.04	5.0
Onions, Spanish	0.6	0.04	4.1
Parsnips	1.7	0.5	21.1
Peas, green, boiled	4.6	0.6	13.3
Potatoes, new, boiled	1.4	0.01	19.7
old, boiled	1.9	0.02	16.0
Spinach, perpetual	1.7	0.1	2.6
Turnips	1.2	0.1	4.4

FRUITS

Apples, Cox's Orange	0.3	0.2	12.5
Bananas, Canary	1.6	0.1	27.4
Oranges, South African	0.8	0.1	8.8
Pears, William	0.3	0.1	8.1
Plums, Victoria	0.4	0.3	10.3

CEREALS

Bread, white	6.3	0.1	47.1
Flour	11.1	1.3	76.1
Oats, rolled	13.1	6.5	69.5
Rice	6.5	0.4	80.8
Shredded wheat	9.8	1.1	77.7
Macaroni, stick	12.8	0.2	75.5

SUGARS, ETC.

Cocoa, average	18.1	26.8	40.3
Golden syrup	0.3	—	76.4
Jam, strawberry	0.3	—	66.4
Marmalade	0.2	—	68.6
Sugar, lump, granulated, etc.	—	—	100.0

it is sufficiently exact to take these average values. We should mention that in the value given for proteins we have allowed for the heat of combustion of the urea that would be formed from them during metabolism in the body. On *complete* combustion to carbon dioxide, water and nitrogen, proteins yield on an average 5.6 Calories per grm.

In this way then the reader can easily determine the number of Calories supplied to his body daily. But to decide whether this is an adequate energy supply it is necessary to determine the amount of energy actually expended by the body per day. This will not necessarily be equal to the amount of energy supplied per day because the body has abundant provision for the storage (as glycogen or fat) of fuel taken in in excess, and it can produce energy from these reserves or even at the expense of its own substance during a period when the immediate fuel supply from external sources is inadequate. The total energy being produced in the body at any time can, as we have seen, be measured directly by means of the Atwater-Benedict calorimeter, but there are but few institutions that possess such an elaborate piece of apparatus, and in any case it is usually more convenient to investigate the energy production by a person carrying out a piece of work under ordinary conditions of practical life by making measurements of the amounts of the end-products of combustion that he is producing. As we have seen, the circumstance that the law of the conservation of energy applies to the body justifies us in this procedure. What we require for the purpose is a knowledge of the amounts of carbon dioxide and water produced in a given time. The estimation of the carbon dioxide presents no difficulties if we have facilities ~~for collecting~~ and

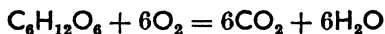
analysing expired air, but the amount of water formed is very difficult to estimate directly as it leaves the body through so many different channels. For this reason the amount of oxygen consumed by the body in the given time is estimated instead; from the amount of carbon dioxide simultaneously produced the fraction of the total oxygen that is used for oxidising carbon is readily calculated; the remainder is evidently the oxygen used for the oxidation of hydrogen so that from this the actual amount of hydrogen oxidised at once becomes known. The problem of determining the total energy produced in the body during any time interval thus resolves itself into a determination of the oxygen consumed and the carbon dioxide produced during that interval; into a determination, that is, of the **total respiratory exchange**.

In this way then we can determine the amount of carbon and of hydrogen being oxidised in the body per minute. But in order to calculate from this the amount of energy being produced we must take into account the circumstance that the body is not oxidising elementary carbon and hydrogen as such, but is oxidising the carbon and hydrogen contained in the molecules of carbohydrates, fats and proteins. From the data of our total respiratory exchange determinations, then, we must calculate the amounts of carbohydrate, fat and protein actually being burnt in the body and then work out the number of Calories of energy by using the heats of combustion of these materials as given on p. 225. This can be done by taking advantage of the fact that owing to the different ratios of carbon to hydrogen in these substances the proportion of the total oxygen used in oxidising them that goes to form carbon dioxide will vary according to the particular substance or substances being oxidised. Since any given volume of

carbon dioxide contains exactly its own volume of oxygen, the volume of carbon dioxide produced is equal to the volume of oxygen used to oxidise the carbon, so that the ratio

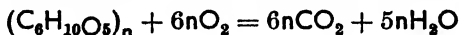
$$\frac{\text{volume of carbon dioxide produced}}{\text{total volume of oxygen used}}$$

will tell us the proportion of the total amount of absorbed oxygen that is being used for oxidising carbon. This ratio is termed the **respiratory quotient**. Let us illustrate by means of examples the way in which a knowledge of the value of this quantity enables one to deduce the kind of material that is undergoing oxidation in the body at any moment. Consider first of all the case of glucose. Whatever the intermediate stages, its ultimate oxidation to carbon dioxide and water may be represented by the equation:



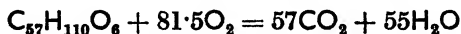
From this it will be seen that for each molecule of oxygen used up, a molecule of carbon dioxide is formed; therefore, since equal numbers of molecules are contained in equal volumes of all gases, for each cubic centimetre of oxygen utilised, a cubic centimetre of carbon dioxide is evolved. In other words, since the glucose molecule already contains enough oxygen to account for the oxidation of its hydrogen atoms to water, the amount of oxygen which must be supplied from outside, in order to oxidise glucose completely, is equal to that which would be required for combination with the carbon. But of course it must not be supposed that the actual oxygen atoms originally contained in the glucose molecule appear exclusively as water, and that none of them go to form carbon dioxide. We have represented the relationships in this way merely for

convenience in estimating the amount of extra oxygen required for the complete oxidation of the sugar. And seeing, as we have said, that the volume of oxygen required for the production of a given volume of carbon dioxide is equal to that of the carbon dioxide itself, it follows that if glucose—or, indeed, any other carbohydrate—were the only fuel undergoing oxidation in the body at any moment, the respiratory quotient would be equal to 1. Actually in our studies of the metabolism in muscle we saw that the primary carbohydrate fuel of the body is glycogen; but if we write its oxidation as



we see at once that in this case also the respiratory quotient is exactly unity.

Next, let us consider the case of fats, as represented by tristearin (p. 124). The total formula obtained by adding up all the atoms of carbon, hydrogen and oxygen atoms in the molecule of this substance is found to be $C_{57}H_{110}O_6$, so that its ultimate oxidation is to be represented thus:—



In this case we observe, first, that the fuel contains very little oxygen in itself: the molecules of the fats are composed almost entirely of carbon and hydrogen. It is for this reason that a gram of fat gives out more energy on combustion than the same weight of carbohydrate or protein; the fat is composed almost entirely of combustible material, whereas the carbohydrate contains large quantities of oxygen, and the protein of nitrogen. Further, on account of the relatively small proportion of oxygen contained in the molecule, there is much less than would be necessary to oxidise more than a small fraction of the

carbon and hydrogen atoms of the fat: oxygen has therefore to be supplied in sufficient amount to oxidise practically the whole of the carbon and hydrogen of the molecule. So that if fat alone were being oxidised in the body, only a certain fraction of the total quantity of oxygen absorbed by the body would appear in the form of carbon dioxide in the expired air, the remainder going to form water. The ratio of the total volume of oxygen absorbed to that of carbon dioxide evolved would, therefore, be less than 1. We can calculate, from the equation just given, what the exact value of the respiratory quotient in the case of the combustion of tristearin would be. For of every 81.5 volumes of oxygen used for the oxidation, 57 volumes appear finally as carbon dioxide; the respiratory quotient, if tristearin alone were being oxidised in the body, would therefore be $\frac{57}{81.5}$, which is almost exactly 0.7.

Knowing then that the respiratory quotient for carbohydrates is 1 and for fats 0.7, it is quite easy, in any case where we can assume that these are the only classes of substances being utilised as fuel in the body, to calculate from the observed respiratory quotient, the relative weights of each actually being oxidised.

In order to make the method of doing this quite clear we will consider a definite numerical example:—

Suppose that in a given determination the respiratory quotient of a subject was found to be 0.95.

Let x litres stand for the volume of oxygen he uses for the oxidation of fat in a given time,

and y litres represent the volume of oxygen he uses for the oxidation of carbohydrate in the same time.

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Then his total oxygen consumption during this time is $(x + y)$ litres.

Since the respiratory quotient for fat is 0.7 the volume of CO_2 produced from fat in the given time will be $0.7x$ litres.

And similarly the respiratory quotient for carbohydrate being 1.0 the volume of CO_2 produced during this time from carbohydrates will be $1.0y$ litres.

The total CO_2 production during the time considered will therefore be $(0.7x + 1.0y)$ litres.

The subject's respiratory quotient will therefore be represented by

$$\frac{0.7x + 1.0y}{x + y}$$

and this, we are told, was found experimentally to be 0.95.

This gives us an easy equation from which it at once follows that $y = 5x$; or, in words, for each litre of oxygen that the subject uses for the burning of fat he uses 5 litres for the burning of carbohydrate, i.e. of every litre of oxygen absorbed $\frac{5}{6}$ l. goes to oxidise carbohydrate and the remaining $\frac{1}{6}$ l. to oxidise fat.

Now from the equations we have already given (p. 230) for these oxidations we see that 6 gram-molecules, i.e. 6×22.4 l. of oxygen are required for the oxidation of each $\text{C}_6\text{H}_{10}\text{O}_5$ unit, i.e. 162 grms. of glycogen.

$\therefore \frac{5}{6}$ l. of oxygen oxidise $\frac{5}{6} \times \frac{162}{6 \times 22.4} = 1.004$ grms. of glycogen, which at 4.2 Calories per gram. (p. 225) yields practically 4.22 Calories.

Similarly, assuming that the fat being oxidised is tri-stearin, it is seen that 81.5×22.4 l. of oxygen oxidise 1 gram-molecule, which adds up to 890 grms. of the fat.

$\therefore \frac{1}{8}$ l. of oxygen oxidises $\frac{1}{8} \times \frac{890}{81.5 \times 22.4} = 0.081$ grm. of fat

which at 9.3 Calories per grm. (p. 225) yields 0.76 Calories.

\therefore total energy produced per litre of oxygen utilised at R.Q. 0.95

$$= 4.22 + 0.76 = 4.98,$$

i.e. in round numbers 5, Calories per litre of oxygen utilised.

In an exactly similar way it is possible to calculate the energy liberated on utilisation of 1 litre of oxygen at any other respiratory quotient. An interesting case is that of respiratory quotient 0.85, which is just half way between 0.7 and 1.0. In this case as the reader will readily confirm for himself, the volume of oxygen used for the oxidation of carbohydrate is equal to that used for the oxidation of fat, so that these materials are being utilised in the proportion of $\frac{162}{6 \times 22.4}$ to $\frac{890}{81.5 \times 22.4}$ which works out to be 2.47 parts of carbohydrate (glycogen) for every 1 part of fat.

In actual practice, however, it is quite unnecessary to go through these calculations whenever a determination of total metabolism is made. The Calorie equivalent of 1 litre of oxygen at each value of the respiratory quotient has been worked out once for all by the same method as we have just illustrated in our example, and the results are available in tables. They naturally vary slightly according to the exact values taken for the heats of combustion of glycogen and fat: the values given by Zuntz and Loewy are as follows:—

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R.Q.	Calorie equivalent of 1 litre oxygen used for oxidising a mixture of glycogen and fat
0.71	4.795
0.75	4.829
0.80	4.875
0.85	4.921
0.90	4.967
0.95	5.012
1.00	5.058

It will be noticed that when glycogen alone is being oxidised a round 5 Calories are obtained for each litre of oxygen absorbed, but somewhat less than this as the fuel contains a higher proportion of fat. Now under the conditions that are generally chosen for the determination of a subject's total energy production it is usually justifiable to assume as we have done in the above calculations that the body is running on a mixture of carbohydrate and fat only; that the amount of protein being oxidised as such is negligible. In fact it is usually arranged that this shall be so by carrying out the determination so long after the subject's last meal that there has been time not only for the complete digestion and absorption of the constituents of that meal, but also for the deamination and conversion into carbohydrates of the absorbed amino-acids. Hence the reason why determinations of total metabolism are usually carried out in the morning before the subject (but not necessarily the experimenter) has had his breakfast. In any given case, however, where it is necessary for reasons of exactness or otherwise to take into account the amount of protein burnt in the body in a given time this can be done by estimating by Kjeldahl's method the total amount of nitrogen excreted in the urine during that time. From the average composition of proteins we know that this amount of nitrogen must

have been derived from the decomposition of an amount of protein equal to 6.25 times the weight of nitrogen excreted, and from further knowledge of the composition of proteins we can work out how much oxygen would be required for the oxidation of the amount of protein thus calculated to have been burnt, and how much carbon dioxide would thereby be produced. (The respiratory quotient of a protein is found in this way to be approximately 0.8, and each grm. of nitrogen excreted corresponds to the usage of 5.9 litres of oxygen and the production of 26.5 Calories of energy.)

Subtracting now from the total carbon dioxide produced and oxygen used by the subject the amounts of these gases associated with the protein metabolism we obtain a "non-protein" respiratory exchange and a non-protein respiratory quotient, from which we can calculate the amounts of carbohydrate and fat also being oxidised, in exactly the same way as in the case where the protein metabolism could be neglected. We are then in possession of a complete knowledge of the amounts of protein, fat and carbohydrate undergoing oxidation in the subject's body, of the amount of energy furnished by each of these materials, and so of the total energy production during the time of the observation.

Now the total energy produced by a subject in a given time may be conveniently regarded as made up of two portions. In the first place there is the energy required to keep the body continuously at a temperature of 37° C. and to maintain such essential processes as the heart beat and respiratory movements. This amount of energy is referred to as the **basal metabolism** as it represents the "base line" below which the energy consumption cannot fall if life is to be maintained; it is as it were the amount

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of energy consumed in keeping the engine just "ticking over." In addition to this the total energy turnover will include the energy equivalent of any external physical work that the subject performs. With regard to the basal metabolism, seeing that it is concerned with the maintenance of the body temperature, it is evident that its amount will depend in the case of any individual on the ease with which heat can escape from his body, and this will depend primarily on the extent of his body surface. So that while we should not expect the basal metabolism to be the same for all subjects, we should expect an approximately constant metabolism per unit of body area, and this, indeed, is found to be the case in all normal individuals. An average man has been found by various methods to have a body area of about $1\frac{3}{4}$ square metres; that of an average woman is slightly less—about $1\frac{1}{2}$ square metres—while an area of approximately 1 square metre is possessed by a child of 10 years. The normal rate of basal metabolism is found to be approximately 40 Calories per square metre per hour, so that for 24 hours the average man would require $40 \times 1.75 \times 24$ Calories, i.e. 1680, or in round figures 1700 Calories, for basal purposes. In women the figure amounts to only 1400 Calories: they are more conservative of energy than men. It is hardly necessary to point out that this figure applies to an individual in comfortably warmed surroundings and that any circumstance that increases the rate of cooling of the body will necessarily correspondingly increase this figure. It is interesting to observe that this figure of 40 Calories per square metre per hour applies not only to humans, but also with relative small variations to the whole range of mammals. But this is not surprising when one bears in mind the fact that all mammals have practically the same

body temperature. Of course the total metabolism per unit weight in the different mammals will be widely different because as the size increases the proportion of the body that is in the surface and so exposed to cooling becomes relatively so much smaller. The following table (from Voit and Rubner) will illustrate these points:—

Animal	Weight (kg.)	Calories produced per 24 hrs.	
		per kilo. of body weight	per sq. m. of body surface
Horse	441	11.3	948
Pig	128	19.1	1078
Man	64.3	32.1	1042
Dog	15.2	51.5	1039
Mouse	0.018	212	1188

But that other factors than mere area must be taken into account is shown by the fact that shearing a sheep doubles its rate of metabolism—a result that indicates that the sheep depends much more on its coat for the regulation of its heat loss than on adjustment of the blood supply to the vessels of the skin, which is a factor of prime importance in ourselves.

Returning now to a consideration of the conditions in man we should point out that quite apart from its purely scientific interest this determination of the basal metabolism has an important medical application inasmuch as the rate of basal metabolism is much affected by the products sent into the blood by the glands of internal secretion. In this way the determination of the rate of basal metabolism is of great value in the clinical diagnosis of disturbances of the endocrine balance of the body. The most striking case in point is furnished by the thyroid whose internal secretion thyroxine (p. 102) has a very marked stimulating action on the general metabolism. In **simple goitre**, which is an enlargement of the gland produced by a lack of iodine, the amount of thyroxine

produced is below normal, and the basal metabolism is correspondingly reduced in rate, an effect that is also to be seen in the lethargic disposition of the patient. On the other hand in **exophthalmic goitre** the thyroid secretion seems to be abnormally increased and possibly of altered composition, with the result that the basal metabolism may be increased up to twice its normal rate with a corresponding degree of restlessness in the patient. In the case of variations in the activity of the pituitary there are also concomitant changes in the rate of metabolism, but in this case the departure from the normal value is usually not more than 20 per cent. Of course in all these cases it is not the total metabolism of the particular patient that is of significance, but the metabolism in Calories per square metre of body surface. In order to calculate this, however, it is not necessary in these days to measure directly the body area of the subject. So many such measurements of body areas have by now been made that the statistical relation between the body area and the weight and height is well known. The body area of any individual may therefore now be obtained from his weight and height either by means of the formula

$$A = W^{0.425} \times H^{0.725} \times 0.007184$$

where A is the required area in square metres,

W is the weight in kilos.,

and H is the height in cm.,

or by reference to tables or graphs where all the possible cases have been worked out once for all.

Before we proceed further with this discussion of energy exchanges of the body it should be mentioned that there are cases where these considerations will not apply; where, owing to secondary disturbing factors, the

respiratory quotient is not a safe guide to the nature of the materials being oxidised in the body. In a diabetic patient, for example, the respiratory quotient is usually much lower than corresponds with the processes of oxidation actually taking place. This is partly on account of the excretion of acetoacetic and hydroxybutyric acids, the formation of which involves a consumption of oxygen without corresponding production of carbon dioxide, and partly also on account of the abnormally great rate at which amino-acids are being converted into sugar. This change also involves a consumption of oxygen since the sugar contains a relatively greater proportion of oxygen than do the amino-acids. But this sugar escapes in the urine; it is not burnt with the formation of a corresponding amount of carbon dioxide, so that for this reason again the ratio $\frac{\text{CO}_2}{\text{O}_2}$ is reduced.

Somewhat similarly a hibernating animal such as a dormouse or marmot takes up during the winter oxygen that it does not use immediately for purposes of oxidation, but for the conversion of its stored body fat into glycogen—and this to such an extent that the weight of the animal actually increases, although during hibernation it consumes no food. The result, again, of this absorption of oxygen without corresponding carbon dioxide production is to give values of respiratory quotient as low even as 0.3. On the other hand, during the summer the animal replenishes its body fat from carbohydrate food. This latter contains a greater proportion of oxygen than the fat to which it gives rise, so that less oxygen need be absorbed from the air than corresponds to the rate at which actual processes of oxidation are going forward in the body, and, as a consequence, the ratio $\frac{\text{CO}_2}{\text{O}_2}$ tends to

be high and may exceed unity. From these examples it will be realised that the respiratory quotient can be justifiably used to determine the nature of the substances being oxidised in the body only when it can be assumed that these oxidations are going to completion to carbon dioxide and water, and are not being arrested at any intermediate stage.

Returning, however, to the much more usual case where this assumption is justified, we will mention that we have explained this calculation of energy output from total respiratory exchange so fully because it is so commonly carried out. It is only if one is a wealthy patient in a particularly well-equipped hospital that one has one's energy production measured directly in a calorimeter of the Atwater-Benedict type. The method of indirect calorimetry is almost invariably employed because it is so much simpler to make an air-tight chamber for the measurement of the total respiratory exchange than it is to make a heat-tight chamber for measuring the energy output directly. In describing the Atwater-Benedict chamber we have already mentioned one type of apparatus employed for the estimation of the total respiratory exchange, but we ought to mention other simpler arrangements that are more particularly suitable for this purpose in experiments of relatively short duration. In these it is usually more convenient to estimate the amounts of carbon dioxide and oxygen concerned by the ordinary methods of volumetric gas analysis than by weighing as in the Atwater-Benedict apparatus. For the most accurate estimations of the total respiratory exchange it is usual to enclose the subject or patient in an air-tight respiratory chamber through which a current of air is drawn by means of an accurate gas meter (a gas meter when rotated

by a motor acts as an air pump). From the number of revolutions of the meter and its dimensions the amount of air leaving the chamber can be very exactly known. An average sample of this outgoing air is collected and analysed as is also a sample of the air entering the chamber—this will, in general, of course, be atmospheric air. It is not necessary to measure directly the volume of air entering the chamber: this will always be somewhat greater than the volume of the outgoing air, except in the case where the subject happens to give to the air current a volume of carbon dioxide exactly equal to that of the oxygen he absorbs, i.e. in the case where the respiratory quotient is exactly 1.0. Its exact amount can be calculated on the obviously justifiable assumption that the *nitrogen* passes through the chamber unchanged in amount, so that knowing, as we do, the total volume of nitrogen leaving the chamber and the *percentage* of nitrogen in the ingoing air, it is easy to calculate the volume of ingoing air in which the same total volume of nitrogen must have been contained. In this way one gets to know the amounts of oxygen and of carbon dioxide entering the chamber and also the amounts of these gases leaving the chamber in a given time, so that the amount of oxygen absorbed and the amount of carbon dioxide produced by the subject become known. A second method for determining the same data, and one that, while being not quite so exact, requires a much less elaborate outfit, consists in causing the subject to breathe through valves so arranged that the whole of the expired air during a given period is collected in an air-tight rubber-lined canvas bag, known after its inventor as a Douglas bag. A small sample of this expired air is analysed and the total volume of it is estimated by

squeezing it out of the bag through a gas meter. The air inspired by the subject is also analysed and its volume is calculated from that of the nitrogen in exactly the same way as we have just described. In fact the only essential difference between these two procedures is that in the bag one collects and analyses pure expired air and not a mixture of expired and unchanged air as in the chamber method. For clinical purposes the determination of the total respiratory metabolism has been very much simplified by an apparatus designed by Krogh. Realising that particularly in the case of patients not accustomed to respiratory experiments excitement often leads to increased respiratory movements and so to a washing out of some of the loosely-combined carbon dioxide that is present in considerable quantities in the blood, with the result that the observed carbon dioxide output may be much greater than the amount of carbon dioxide actually produced in the body by oxidation, he fixes the respiratory quotient at a known value by giving a diet of known composition on the day before the determination (which is easy to control in a hospital patient) and, assuming that this is the material that is undergoing oxidation at the time of the experiment, i.e. before breakfast next day, he simply measures the rate of oxygen consumption (which is not affected by over-breathing), and knowing by means of the table on p. 242 the Calorie value of each litre of oxygen at the respiratory quotient he has chosen he can at once calculate the energy output. The spirometer used for this determination of oxygen consumption is of the simplest kind. It consists of a floating gas holder whose movements are recorded on a drum. The gas holder contains a tray of efficient soda-lime for the absorption of carbon dioxide; it is filled with oxygen at the

commencement of the determination, and the subject breathes in and out of it through a simple mouthpiece, no valves being necessary. As the oxygen is consumed, the record of the respiratory movements sinks lower and lower on the drum, and from the rate of slope of the record the rate of oxygen consumption can at once be obtained from the calibration figure of the instrument.

Coming now to the question of the energy expenditure during muscular work it is evident that the same general methods can be applied to its determination as are used for the resting subject. A man can ride a stationary bicycle-ergometer inside a calorimeter and, as we have seen, the law of conservation of energy may in this way be shown to apply equally during the work as during a period of rest. Or a respiratory chamber may be constructed of such size as to accommodate the bicycle, and the total respiratory exchange occurring during the performance of a measured amount of work can be measured, and from it the overall efficiency of the body as a machine. But it more commonly happens that it is the energy production of the body during a spell of some ordinary everyday activity carried out under the usual working conditions that is to be measured. In this case it is often not possible for the work to be performed in a calorimeter or respiratory chamber: some form of apparatus must therefore be used to which the subject may be connected while he carries out the work. For outdoor activities such as walking, running, or climbing, a Douglas bag carried on the man's back can be used. For more or less sedentary work Benedict places not the whole subject, but only his head in a respiratory chamber made from an ordinary pail; over the open end of the pail a large rubber bathing cap is tied; in this is cut a hole large enough to

admit the subject's head but small enough to make an air-tight joint round his neck; a small window let into the pail enables the subject to see, and two tubes soldered into the sides of this "helmet" connect it to a respiratory circuit.

By methods such as these the total respiratory exchange, and so the energy involved in a large number of human activities has been measured. It would be beside our point to go into the details of the results obtained, but we might just indicate their general trend. As we have seen the basal energy expenditure of a man at rest in comfortably warmed surroundings is about 1700 Calories per 24 hours. Any physical activities the man indulges in will involve an equivalent addition to this basal amount, in proportion to the severity of the effort. For a man engaged in a sedentary occupation at home this will mean an additional 500 Calories or so, so that the daily energy requirement of such a man will be about 2200 Calories. On the other hand, work of the severity of gardening involves a total energy output of about 4000 Calories a day; while the record for ordinary occupations seems to be held by lumbermen who perform very hard physical work in cold northern climates and have a daily energy turnover of over 8000 Calories, although even this figure has been beaten under experimental conditions by an enthusiastic rider on the bicycle ergometer. The average for the whole population with its multifarious pursuits amounts to 3000 Calories per head per day. It is to be noted in this connection that it is only the physical work a man does that influences his energy expenditure. Benedict showed in his calorimeter that mental work produces no measurable increase of metabolism—a peculiarly fortunate circumstance that doubtless explains the frequency with

which in the past intellectual triumphs have been achieved under conditions of semi-starvation. The creative effort is not controlled by the laws of thermodynamics.

From figures such as these and the table of the composition of foods it is of course quite easy to work out an adequate diet for an individual of given size employed in any particular type of work. Of course, the diet must not only yield the required number of Calories in the body, but must include at least the minimum amount of protein necessary for the maintenance of nitrogenous equilibrium (Chapter VI) and must have a sufficient proportion of carbohydrates to fat to prevent the formation of the acetone bodies (Chapter XII). It must also provide the necessary amounts of inorganic salts, and the vitamins of which we shall speak later (Chapter XV).

When such a diet has been worked out, it is usual to allow some 10 per cent. more than the calculated quantity per day, not only in order to cover the work involved in the secretory and mechanical processes of digestion, but also because the absorbed products of digestion — particularly the amino-acids derived from proteins — have a stimulating action on the general oxidative processes of the body. This phenomenon is known as the **specific dynamic action** of foods. As it is most marked in the case of the amino-acids derived from digested proteins, and as in metabolism experiments it is usual to take the subject in a post-absorptive condition, the energy lost in this specific dynamic action will not have been included in the estimation of the total energy requirement.

At present we are uncertain as to the reason for this behaviour. It may be that the deamination of the amino-acids requires the expenditure of energy in the tissues where this process goes forward, or it may be that

either the amino-acids themselves, or internal secretions formed from them, exert a direct stimulating influence on cell metabolism, and cause the process of oxidation of fats and carbohydrates to proceed more vigorously. But whichever explanation comes to be accepted finally, the observation that in the presence of excess of amino-acids the combustion of *other* constituents of the cell protoplasm is accelerated, is well founded. The extra energy of this more rapid oxidation, not being required for the performance of mechanical work, is lost from the body as heat.

It is evident that so complete a knowledge of the combustion processes occurring in the tissues as is given by these studies of the total respiratory exchange must be of first-rate importance in the study of the working of the body as an energy-producing machine. In Liebig's day the view was current that protein alone was capable of supplying mechanical energy when oxidised in the muscles, the oxidation of carbohydrates and fat serving merely to produce heat for the maintenance of the temperature of the body. But this view remained prevalent only until accurate methods were elaborated for the estimation of nitrogen in the urine. It was then found that the performance of a measured amount of work was accompanied by the oxidation of much more material than corresponded with the amount of protein that would have been required to furnish the observed total amount of nitrogen excreted during the work. The classical experiment on this subject was performed as long ago as 1865 by Fick and Wislicenus, who measured their respective outputs of nitrogen during an ascent of the Faulhorn. They calculated the amount of protein to which this nitrogen corresponded, knowing the average percentage of nitrogen in proteins. For Wislicenus this

came to be 37 grms. We already know that the amount of energy to be obtained by the oxidation of 1 grm. of protein in the body is 4.3 Calories, and since each Calorie is equivalent to 425 kilogram-metres of work, the total amount of energy that Wislicenus could have obtained from the oxidation of his protein was $37 \times 4.3 \times 425 = 68,000$ kilogram-metres (approximately). Now Wislicenus himself weighed 76 kilos., and during the climb he pulled himself through a height of 1956 metres. He therefore did 76×1956 , i.e. 148,656 kilogram-metres of work. It is evident that, even assuming, as we have done, that the whole of the energy of oxidation of the proteins was converted into mechanical work, i.e. that the body is 100 per cent. efficient as a transformer of energy—which it certainly is not—not half of the total energy expended in the climb can be accounted for; so that a considerable fraction must have been derived from non-nitrogenous materials.

While it is thus evident that both carbohydrates and fats are *ultimately* used as fuel for the production of mechanical energy in the body, the question as to whether both of these fuels can be utilised directly by the muscles (as carbohydrate certainly is) or whether the fat is first converted elsewhere into carbohydrate is not so easy to answer. Of course, in whichever way the fat were utilised—direct or indirect—the general respiratory quotient of the body would be the same; no mere measurement of respiratory quotient would enable us to decide between these possibilities. It was hoped at one time that the problem could be solved by comparing by means of total respiratory exchange measurements the efficiency with which a subject performs muscular work on a diet composed mainly of carbohydrates with his efficiency on a

diet including a high proportion of fat. The idea behind these investigations was that if fat is used directly as a fuel by the muscles the efficiency of its utilisation, that is the number of Calories of energy that must be produced in the body in order that a given amount of mechanical energy shall be produced, would probably be the same as during the utilisation of carbohydrates, but that if the fat is first converted into carbohydrates outside the muscles, the energy of this conversion, which amounts to 23 per cent. of the total heat of combustion of the fat, would be lost as heat and thus would not appear as mechanical work, so that the efficiency of the body as a machine would be 23 per cent. less on the fat than on the carbohydrate diet. Actually it was found that the body was less efficient on the fat diet, but to the extent of only 11 per cent., so that from these experiments our question does not receive a definite answer. More recently, however, it has been found that for the excess metabolism during a short bout of not too severe exercise, i.e. for the additional metabolism over and above the resting metabolism, the respiratory quotient is often practically unity. This would indicate that the muscles are running exclusively on carbohydrate fuel. But when the exercise is prolonged the respiratory quotient of the excess metabolism falls below unity showing that fats are (directly or indirectly) being utilised. If, however, the effort is severe the apparent excess respiratory quotient rises above unity during the work on account of the expulsion of the loosely-combined carbon dioxide of the blood by the lactic acid diffusing from the muscles; it afterwards shows a corresponding low value during the recovery period while the carbon dioxide, instead of being expelled, is being used to replenish the normal store of

this substance in the blood. We cannot discuss these matters at greater length here, but from this bare outline it will probably be abundantly evident that many complications enter into the interpretation of the respiratory exchanges of the body during work, and that at first sight simple production of energy by the combustion of fuel in the human machine gives rise to many questions to which at present we have but incomplete answers.

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CHAPTER XIV

ENZYMES AND OXIDATION CATALYSTS

"A little leaven leaveneth the whole lump."—*St. Paul.*

THE reader must have noticed that at many points in the foregoing account of the chemical changes occurring in the body we have described these changes as being brought about under the influence of agents we have called ferments or enzymes; this is especially obvious in our description of the changes occurring during digestion, where we described each step as being brought about under the influence of a particular enzyme that formed a characteristic constituent of the digestive juice concerned; and in many cases we have been led to a knowledge of the chemical functions of an organ or of the course of metabolism within it by a study of the enzymes it contains. But so far we have not turned aside from our main argument to give any account of the nature of these enzymes or of the general characteristics of their activities. In this chapter, therefore, we shall attempt to make up this deficiency.

The changes occurring in sugary solutions such as fruit juices when these are allowed to stand without special precautions and which lead to the formation of alcohol in the liquid and the bubbling off from it of carbon dioxide, thus giving rise to a spurious appearance of boiling known as fermentation (Lat. *fervere* = to boil), have been known to man ever since the dawn of antiquity. The Biblical record ascribes the discovery to Noah, and ever since his

remote age the process has been utilised on the one hand for the production of gas wherewith to bring about the "rising" or leavening of the bread of man's food, and on the other as the basis of the manufacture of the wine with which his heart is gladdened. With the development of biological science it finally became realised after much struggle and discussion that such processes of fermentation and putrefaction are invariably brought about by micro-organisms such as yeasts or bacteria that had gained access to the fermenting or putrefying material, the final proof that sterile material exhibited no such changes being provided by the researches of Pasteur. The principle thus finally established was, as is well known, at once applied by Lister in the development of modern aseptic surgical technique.

Long before this, however, digestive juices had been collected and studied and it had become evident that the actions produced by them were very similar to the fermentations set up by micro-organisms. There thus came to be distinguished two types of ferments: those, such as yeast and bacteria, that were really living organisms—these were termed "organised" ferments—and those such as the ferments of the digestive juices that were merely substances that could produce their actions in test-tubes outside the body and in the absence of living matter—these received the name of "unorganised" ferments. Then the idea gradually formed itself that even the living organisms previously designated organised ferments acted by virtue of unorganised ferments that they contained, and eventually the name **enzymes** (from the Greek *ἐν ζύμῃ* meaning "in yeast," on account of the occurrence therein of the most familiar example) was coined to include all of these active agents whether

known to act in a digestive juice outside the body or only within the confines of a living cell. The most striking proof that the chemical changes brought about by living cells are really brought about by means of enzymes contained within those cells was furnished when in 1897 Buchner succeeded in pressing out from ground-up yeast a juice that would bring about the conversion of sugar to alcohol and carbon dioxide although, as he carefully proved, the liquid was quite free from intact yeast cells. To the enzyme thus extracted Buchner gave the name **zymase**—again from the Greek for yeast. It is evident that the yeast plant uses this zymase in order to bring about the fermentation of the sugar and so to obtain energy for its life processes.

When once it had thus been realised that the most characteristic of all ferment actions, namely, the production of alcohol and carbon dioxide from sugar, is brought about under the influence of an enzyme that could be extracted and made to act in the absence of living matter it was soon found to be possible to obtain from other micro-organisms and also from tissues and body fluids extracts containing the enzymes characteristic of these various materials, and so we come to conceive of enzymes as agents universally present in living matter and responsible for the carrying on of the chemical changes on which the continued existence of the organism depends.

In some cases it is possible to precipitate the active agent from such extracts by means of alcohol, and so to obtain a dry preparation containing the enzyme. Such preparations usually give the colour reactions for proteins, but as they undoubtedly do not consist of the pure enzyme itself it is difficult to decide whether the enzymes themselves are all protein in nature or whether they are merely

contaminated in these preparations with proteins of the tissues from which they were extracted. In some cases it has even proved possible to obtain a crystalline preparation—for example, the enzyme urease has been obtained in the form of crystals giving the reactions of a globulin, but even here we are uncertain as to whether the protein is an essential part of the enzyme structure or merely an admixed impurity accompanying it.

Seeing, then, that we do not know enzymes as pure substances, it is evidently impossible to give them systematic chemical names; under these circumstances it has been found most convenient to name each enzyme, or, in many cases, each type of enzyme after the substance on which it exerts its action. The termination “-ase” is usually added to the name of the substrate in order to form the name of the enzyme. Thus, an enzyme that hydrolyses starch is called an **amylase** (Lat. *amylum* = starch); a fat-splitting enzyme is known as a **lipase** (Gr. *λίπος* = fat). In order, then, to refer to any particular enzyme, its place of occurrence is usually also mentioned. Thus one speaks of the pancreatic lipase, and so on. But this method of naming ferments has not been applied to a few which were already known before the present nomenclature was introduced. For example, enzymes that attack proteins are still more commonly called “proteolytic enzymes” than “proteases,” and they are usually referred to by names which do not conform to the above rule. Pepsin and trypsin have become such household words in the language of the biochemist that one is reluctant to replace them.

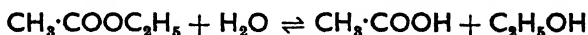
We hasten to point out, however, that our present lack of complete knowledge of their chemical structure has not prevented the accumulation of a great deal of information

regarding the activities of enzymes and the various factors that influence them, and it is to a brief consideration of these topics that we must now address ourselves.

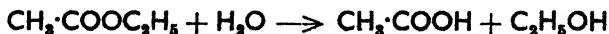
The first striking fact that emerges with regard to enzymes is their enormous potency. Perhaps the reader has already realised that they bring about, in the gentle warmth and under the conditions of mild alkalinity or acidity that prevail in the body, reactions that in the laboratory would require boiling temperatures and strong acids and alkalis. But further, given sufficient time and suitable conditions, a given weight of an enzyme preparation can bring about its characteristic change in a practically infinite amount of its substrate. For example, preparations of amyllopsin have been made from pancreatic juice that have been known to digest almost a million times their weight of starch! Such a fact as this proves very strikingly that enzymes must bring about their reactions without being themselves directly used up in the process—that they are, in fact, catalysts as the chemist would say, and this point of view is consistent with all we know about them. We can thus define enzymes as catalysts elaborated by living matter for suitably influencing the chemical changes on which the continued existence of that matter depends. The validity of this point of view is further enhanced by the circumstance that certain deductions applicable to catalysts in general are found also to apply to enzymes in particular. For seeing that a catalyst remains unchanged in the system it is evident that it cannot have added any energy to the system. Energy could only be obtained if the catalyst underwent some chemical change—for example, an oxidation—whereas, as a matter of fact, observation shows that it remains unaltered.

It therefore follows that the only respect in which

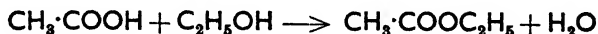
a catalysed reaction differs from that occurring between the same initial substances when left to themselves will be that of velocity. The substances if left to themselves will produce exactly the same equilibrium mixture as in the presence of the enzyme, only much more, often infinitely, slowly. For a change of equilibrium would necessitate a change in the energy content of the system; in order that the reaction might proceed further, for example, it might be that extra energy would need to be supplied. But of furnishing this extra supply of energy a catalyst is incapable, because at the end it retains all the energy it possessed at the beginning of the reaction. This fact has been proved in detail in the case of the hydrolysis of esters—a convenient case to deal with experimentally, as the action even in the absence of an enzyme proceeds at a rate which is sufficiently rapid to be observed with reasonable ease. Suppose, for example, one takes an ester such as ethyl acetate, and leaves it in contact with water for some weeks or months, then it is found that the substance is slowly hydrolysed with the formation of acetic acid and ethyl alcohol:—



The reaction proceeds slowly until a mixture in certain proportions of ethyl acetate, water, acid and alcohol has resulted. No further change then takes place, and the system remains permanently at this equilibrium composition. The reason for this behaviour is found in the fact that in such a system we really have two reverse reactions proceeding at each moment. The one is the hydrolysis of the ester to liberate the free acid and the alcohol:—



The other simultaneous reaction is the reverse of this and consists of the combination of the acid and alcohol to give the ester and water again:—



Now the rate at which each of these reactions proceeds is determined by the concentrations of the reacting substances. At the commencement the concentrations of water and of ester are relatively high, and the hydrolysis proceeds quickly. But as this hydrolysis proceeds, ester and water are being gradually used up so that their concentrations are falling off, and the rate at which the change proceeds becomes progressively slower. But the rate of the re-combination of acid and alcohol, slow at first, is meanwhile gaining in speed, for the concentrations of these substances increase as they are liberated from the ester. The rate of the forward change, then, gradually slows, and that of the backward reaction gradually increases, until there comes a moment when the rates of the two are just equal. At this point equilibrium is attained, and no further change appears to take place because the ester is breaking up just as fast as it is being re-formed. If now we start with the same concentrations of initial substances, but add to the mixture a catalyst such as a little dilute mineral acid, exactly the same kind of result is observed—the same equilibrium mixture is obtained—but in this case much more quickly—in a few hours in fact. This being so, it follows that not only has the catalyst added no energy to the system, but also that it must have accelerated the backward and forward reactions to exactly the same extent, for the final equilibrium condition arrived at depends on the *ratio* of the actual backward and forward velocities, and it is only if these are increased each

in the same proportion that the ratio remains unaltered. From a slightly different point of view this is equivalent to saying that no matter whether we start with a mixture of ester and water, or of acid and alcohol, we shall ultimately obtain the same final mixture of all four substances, both in the presence of the catalyst, when the equilibrium will be attained rapidly, and in the absence of the catalyst, when the same result will be achieved much more slowly.

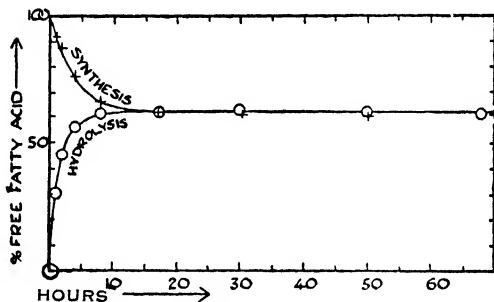


FIG. 11. Reversibility of the action of lipase of castor oil seeds on a mixture of glycerol and oleic acid ("synthesis") and on triolein ("hydrolysis") (from the data of Armstrong and Gosney).

The important thing to notice now is that this has been proved to be the case not only when the reaction is accelerated by such inorganic catalysts as mineral acids, but also when it is brought about under the influence of a suitable enzyme. In Fig. 11 we reproduce some of the results that have been obtained in the study of the action of the enzyme lipase obtained, in this case, from castor oil seeds, firstly, on a mixture of oleic acid and glycerol (upper curve), and, secondly, on the ester or glyceride of

oleic acid and glycerol, i.e. the fat olein itself (lower curve). Nothing could be more striking than the way in which these two curves come together and then run horizontally after about 15 hours, thus indicating the production of identically the same equilibrium mixture of glycerol, oleic acid, ester and water, no matter from which side this equilibrium is approached. The direction in which a change occurs in a chemical system depends entirely upon the relative concentrations of the reacting molecules. If any of these be present in large amount, then, by force of numbers, so to speak, they insist on having a share in the chemical reaction, and so on being used up. On the other hand, if any kind of molecule is present only in small concentration, then there is plenty of room for the production of more of it, so that in general the reaction will tend to proceed in such a way that this result is brought about. A catalyst will not influence the direction in which the change tends to occur; it will merely accelerate the change when it does occur. In this respect the catalyst behaves exactly as does the oil on a machine; it accelerates the movement, but does not initiate it or determine its direction.

Now to say that enzymes equally accelerate both the forward and backward reactions in a chemical system that is under their influence is equivalent to saying that enzymes are capable of bringing about not only breaking-down changes, but also the reverse processes of synthesis. Indeed, this is the very point we have just illustrated in the case of lipase. The first example of this synthetic activity of enzymes was discovered in the case of maltase, which will bring about the condensation of glucose molecules, if not to maltose itself, to an isomeric disaccharide. And recently it has been shown that pepsin

has the power of causing at all events a partial recombination of the products of protein digestion with a consequent re-formation of more complex protein material. It is evident in a general kind of way that both these phases of enzyme action are utilised in the organism—the breakdown phase during the digestion in the alimentary canal of the complex constituents of foods to simple absorbable assimilable units, and the synthetic phase in the tissues where from these simple units the complex constituents of living matter are once more built up. So much, then, for a brief notice of the **reversibility** of the action of enzymes.

We now pass on to consider another very characteristic property of these substances, namely, their remarkable **specificity**. It will doubtless already have been realised that a very large majority of the reactions catalysed by enzymes are of the nature of hydrolyses—the splitting of a complex substance into simpler molecules by the simultaneous taking up of the elements of a molecule of water. Think, for example, of the digestion of a fat into fatty acids and glycerol by means of a lipase, of starch into sugar by an amylase, or a protein into amino-acids by means of a protease. But fundamentally similar as these processes may be from the purely chemical point of view the enzymes bringing them about are by no means interchangeable. A lipase will hydrolyse only fats, an amylase only starch, and a protease only proteins. But in these examples we see instances of enzymes each of which is capable of acting on any member of its own appropriate group of chemically related substrates. In other cases, however, a much more marked degree of specificity is observed, some enzymes being capable of distinguishing between the two optical isomers of a substance—attacking

one rapidly while leaving the other almost, if not quite, unaffected—a circumstance that forms the basis of the biological method of obtaining the more resistant optical isomer from a racemic mixture of the two. An interesting and well-known case of this specificity is furnished by the enzymes that attack the esters of the two forms of glucose, the α - and β -glucosides, in which there is a difference of space configuration only in the end group of the chain. Maltase obtained from malt readily hydrolyses the α compounds (including maltose, which is glucose α -glucoside), but not the β compounds, while **emulsin**, an enzyme present in bitter almonds, hydrolyses the β but not the α compounds. These properties are made use of for obtaining evidence as to whether a given substance is a derivative of α - or β -glucose. A similar case is furnished by the amylases which will attack starch—a complex of α -glucose—but not cellulose which is a complex of β -glucose. It thus comes about that the starch of our food is digested while the cellulose remains as “roughage,” for we possess no enzyme that will hydrolyse β -glucosides. The existence of enzymes with such restricted tastes has suggested the simile that an enzyme must be adapted in structure to the substrate on which it is to act just as accurately as a key must be adapted to a lock that it is to open.

In addition to reversibility and specificity another very well-marked characteristic of enzymes is their sensitivity to heat. It is a universal rule that up to a certain point the rate at which an enzyme acts is increased by rise of temperature, but this, of course, is but the usual effect of increased temperature on the rate of a chemical reaction. But above a certain optimum temperature—at which the rate of the enzyme action is a maximum—

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further increase of temperature leads to a falling-off of the enzyme activity until at the boiling point the action stops altogether. In other words, all enzymes are destroyed on boiling—a circumstance that is very commonly utilised for distinguishing between enzymes themselves and inorganic or other heat-stable catalysts. Bearing in mind this destruction of the enzyme at high temperatures the existence of an optimum temperature for its activity is easily understood. As the temperature is raised the action of the enzyme becomes more and more rapid, but above a certain temperature so also does the rate of destruction of the enzyme by the heat. Ultimately at a sufficiently high temperature so much of the enzyme will have been destroyed that the reduction of effective concentration of the enzyme more than counter-balances the increased activity of the remainder, so that the rate of the reaction is once more reduced.

Then again it is important to mention among the characteristics of enzymes the circumstance that they are very much influenced by changes in the composition of the fluid in which they find themselves. Particularly is this the case with regard to changes in degree of acidity or alkalinity. It is well known, for example, that pepsin will attack proteins only if the solution is strongly acidified with a mineral acid—hence the function of the hydrochloric acid of the gastric juice. Incidentally, it is a matter of some importance in connection with the identification of enzymes that pepsin is not merely inhibited, but is completely destroyed by alkalis, a solution of the enzyme being quite inactive on re-acidification after being made alkaline to phenolphthalein and kept at body temperature for ten minutes. In this way pepsin differs from **rennin**, the milk-clotting enzyme, which is

much less affected by alkalis. On the other hand, trypsin *requires* for its activity the mild degree of alkalinity furnished by the presence of sodium carbonate; it is, however, merely inhibited but not destroyed by acids. The ptyalin of the saliva works best in an approximately neutral solution and is inhibited by both alkalis and acids. In general it is found that for each enzyme there is a very well defined optimum degree of acidity or alkalinity, i.e. an optimum concentration of hydrogen ions at which its activity is a maximum. To a possible explanation of this important phenomenon we shall return when we deal later in detail with the question of acid-base equilibrium and its influence on biological materials and processes (p. 409). Meanwhile we will point out that enzymes are influenced not only by the concentration of acid or base with which they are in contact, but also by the prevailing concentration of other electrolytes as well. This is very well illustrated by the salivary ptyalin which has practically no starch-splitting activity in the absence of chlorine ions. We might at this point remind the reader that many enzymes are secreted as inactive precursors that must come into contact with some activator before the characteristic action is displayed.' In Chapter III we have already explained how the pancreatic trypsinogen which is without action on *native* proteins is activated by direct combination with the enterokinase of the intestinal juice, the fully formed trypsin being composed of a trypsinogen-enterokinase complex. Then again the **thrombin** which converts fibrinogen of the plasma into the insoluble threads of fibrin and so brings about the clotting of blood is not present as such in the circulating fluid, but is formed by the activation of a precursor **prothrombin** when the blood is shed. This last-named

activation is brought about by the action of calcium ions, hence the very common procedures for preventing the clotting of blood for experimental purposes by the addition of oxalate or fluoride which precipitate the calcium in insoluble forms or by citrate which converts it into a non-ionised complex. We should mention that in contrast with this the part played by the calcium that is found to be necessary for the clotting of milk by rennin is not to facilitate the action of the enzyme, but to form a constituent of the clot itself. It forms an insoluble calcium salt with paracasein, which is the product of the action of the enzyme on the casein.

In contrast with these facilitating and accelerating agents we know of many substances that diminish or even completely abolish the action of an enzyme—that “poison” the enzyme as it has been said. The commonest of such enzyme poisons are the compounds of heavy metals such as silver and mercury, cyanides, fluorides and alkaloids; but it is quite certain that substances belonging to such miscellaneous groups affect enzymes in very different ways—a conclusion that is borne out by the fact that these various reagents differ very considerably in their poisonous action according to the enzyme on which they are brought to act.

Substances that exert an inhibitory action on enzymes are also found in the body. For example, the stomach wall protects itself against the digestive action of pepsin by the secretion of an **anti-pepsin** which prevents the pepsin from acting on the tissue proteins. Something similar seems to occur in connection with the clotting of the blood. Owing to the simultaneous presence in it of fibrinogen, and the prothrombin and calcium ions that together would yield active thrombin, the blood would clot

in the vessels were it not for the simultaneous presence of inhibitory substances that prevent the interaction of the prothrombin and the calcium ions to form thrombin. A preparation of such anti-prothrombins is now made from liver, and under the name **heparin** it is extensively used for the prevention of the clotting of blood in animal experiments. The reason why the blood does clot when it escapes from the vessels is that a substance **thrombo-kinase** is liberated from the damaged tissues and the blood platelets and this combines with the anti-prothrombins and so leaves the prothrombin free to be activated by the calcium ions present. This thrombo-kinase, which thus gives the false appearance of acting by directly activating the precursor of thrombin is no longer a mysterious substance of undetermined constitution, but is now known to be identical with the phospholipin kephalin (p. 141). In this connection it is interesting to note that the substance that the leech injects in order to prevent the clotting of the blood on which it feeds acts by directly inhibiting the action of thrombin on fibrinogen and not by preventing its formation. This substance can be extracted from leeches and under the name **hirudin** was formerly much used for experimental purposes as an anti-coagulant.

Lastly, we must briefly mention the methods used for the detection and estimation of enzymes. Not knowing the chemical constitution of any enzyme, we cannot test for it or estimate it as a chemical substance, yet we achieve both of these results by taking advantage of its own characteristic activity. For example, if we wish to test for a starch-splitting enzyme in a solution we add a little starch paste to the solution, and, taking care that the reaction is about neutral, keep the mixture in a warm bath

at body temperature. If then the mixture gradually loses the property of giving a blue colour when a drop of it is treated with iodine, we conclude that an amylase is present in our original solution. And we confirm this conclusion by determining that boiling a portion of our solution leads to a destruction of its starch-splitting power—thus proving that the action was due to an enzyme, since, as we have seen, enzymes are destroyed at a boiling temperature. It is evident that in order that the test shall be carried out conveniently only a small quantity of starch must be added. Otherwise it may be that the time required for an appreciable reduction in the blue colour given with iodine will be excessively prolonged. In a similar way it is in general necessary, in testing for other enzymes, only to use an appropriate small quantity of substrate. In testing for a proteolytic enzyme we investigate whether our given fluid will cause the hydrolysis of a suitable protein added to it. In the case of pepsin it is usual to use fibrin (from a blood clot) stained with carmine. The mixture must, of course, be made acid with 0.4 per cent. hydrochloric acid. If then the fibrin becomes hydrolysed the carmine is liberated into the main bulk of the liquid. As before, a control must be carried out with a previously boiled portion of the fluid in order to prove that the liberation of the carmine was actually due to an enzyme action. The hydrochloric acid itself will not extract the carmine, but alkali will do so, so that carmine fibrin cannot be used in testing for trypsin since for this a medium made alkaline with sodium carbonate is required. In this case fibrin stained with Congo red may be used. Or both pepsin and trypsin may be detected by their digestive powers on casein. For pepsin a dilute solution of casein in hydrochloric acid of

appropriate strength is used; for trypsin a similarly dilute solution made up with sodium carbonate. A little of the solution to be tested is added, and the occurrence of digestion during incubation is detected by the disappearance of the casein, i.e. by the non-appearance of a precipitate of the unchanged protein when on removal of the hydrochloric acid by the cautious addition of sodium acetate solution, or of the sodium carbonate by dilute acetic acid, a test portion of the mixture is brought to the particular degree of faint acidity at which casein is insoluble. Of course, as usual, this must be confirmed by proving in a control test that this proteolytic activity of the solution is destroyed on boiling. A lipase is detected by its power of liberating free fatty acid (detected by the change of colour of an indicator) from a previously neutral emulsion of fat, and in a converse fashion urease is detected by the liberation of alkaline ammonia from urea (p. 68).

The estimation of the degree of enzyme activity is carried out by observing either the amount of substrate acted upon under standard conditions in a given time, or the time required for the complete decomposition of a given amount of substrate. As illustrating the former principle we will mention the old method for the estimation of pepsin. Egg-white is drawn up into capillary glass tubes and coagulated in them by heating. The tubes are then cut into convenient lengths and immersed in the pepsin solution at body temperature for some hours. By experiment it has been found that the length of egg-white digested is proportional to the square root of the pepsin concentration. An example of the second principle is afforded by the usual method for the estimation of amylase, in which the amount of starch digested in a fixed

time is taken as a measure of the amount of enzyme present. This is of some clinical importance, for curiously enough the urine normally contains small traces of amylase that have "leaked" from the pancreas into the blood stream, and so through the kidney. In infective conditions of the pancreas (pancreatitis) the amount of this urinary amylase is enormously increased—hence the diagnostic value of its estimation. This is done by adding a known amount of 1 per mille starch solution to various dilutions of urine, keeping the mixtures at body temperature for half an hour, and then by means of iodine determining in which of the mixtures the starch is just completely digested. From this is calculated the number of c.c. of 1 per mille starch digested in half an hour by 1 c.c. of urine at body temperature. Normally this **diastatic index**, as it is called, comes to be about 10, which is very feeble compared with, say, that of saliva, but in diseased conditions the value may run into hundreds or even thousands.

By this time the reader must be wondering what kind of substance an enzyme really is and what its mode of action that it should be characterised by such remarkable potency, exact reversibility, extreme specificity and such marked sensitiveness to changes in the temperature and composition of its environment. It must be admitted that although we have so far learnt a great deal with regard to these characteristics of enzymes and their actions we have not yet come across any certain clue as to their nature. It is only when we come to study not merely the final products of enzyme action, but the rate at which these products are formed—in other words, when we make dynamic and not merely static experiments—that we obtain information that is capable of giving us any kind

of answer to these fundamental questions. Let us take a specific example. In the presence of a little acid as catalyst cane sugar is rapidly hydrolysed to a mixture of glucose and fructose, and by polarimetric estimations on the reacting mixture at known intervals it is easy to prove that the rate at which the cane sugar is hydrolysed begins to fall off from the moment when the reaction begins, and that, in fact, the rate at any moment is proportional to the ever-diminishing amount of cane sugar left unchanged in the solution at that moment, so that as the amount of sugar remaining unhydrolysed becomes smaller and smaller the rate at which further hydrolysis takes place becomes progressively slower and slower. If we use a greater concentration of the catalyst acid the reaction as a whole takes place at a more rapid, but always at a characteristically progressively diminishing, rate. If, however, instead of using inorganic acid as catalyst we use the enzyme invertase (which, apart from other sources, may be extracted from yeast) to promote the hydrolysis we obtain a very different result. Provided that the amount of sugar is large compared with the amount of enzyme, it is found that the rate of the hydrolysis does not begin to fall off from the moment at which the reaction begins, but remains constant over a relatively long initial period. In other words, the course of the reaction is now represented by a straight line (Fig. 12) instead of by the progressively flatter curve characteristic of the acid hydrolysis. Evidently the rate of the reaction is now not directly dependent on the number of cane sugar molecules present at any moment, for this, just as before, is steadily diminishing, and yet the reaction proceeds at a constant rate. The universally accepted explanation of this and similar observations is that the first step in the action of

an enzyme is the rapid formation of some kind of complex with its substrate, and that it is only the amount of the substrate so combined that undergoes change. If now there is such a concentration of substrate in proportion to enzyme that as soon as one combined molecule of it is

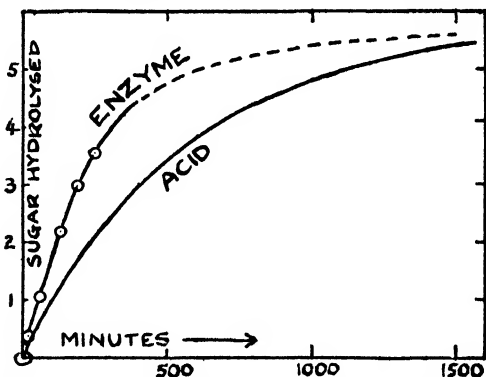


FIG. 12. Rates of enzymic and acid hydrolysis of cane sugar in solutions of equal initial concentrations (5.7 units as measured by the polarimeter). Both reactions reach the same equilibrium point, but while the acid hydrolysis begins to slow off from the beginning the enzyme action maintains an almost constant rate for about 4 hours and then slows off suddenly. (The "enzyme" curve is drawn and the "acid" curve calculated from Michaelis' data.)

decomposed another takes its place the amount of this enzyme-substrate complex will remain constant for a long time, and with it the rate of the reaction. This condition will obtain until so much of the substrate has been decomposed that there is no longer enough of it to keep all the enzyme "saturated"; the concentration of the enzyme-substrate complex then gradually falls off as does consequently the rate of the reaction. It would

be expected from this explanation, that if we start with a mixture containing a relatively small proportion of substrate to enzyme the reaction does not show this initial period of constant velocity, but falls off continuously from the start, there not being enough substrate even at the beginning to saturate the enzyme and convert it all into complex. This is indeed found to be the case. But it must not be supposed that this simple theory covers all cases of enzyme action without difficulty. Many factors have to be taken into account before a really complete explanation of the rate of many enzyme actions can be given. The action may be slowed down by the gradual accumulation of its own products and the consequent increase in the velocity of the back reaction (which, as we know, is also catalysed by the enzyme). Then the products of the action may exert an important influence on the enzyme itself, in some cases of an inhibitory nature similar to the poisoning of enzymes we have already mentioned, and sometimes of an opposite accelerating "auto-catalytic" kind, so that the detailed study of enzyme kinetics is in many cases a very complicated matter. The main point that arises from it is, however, this extremely fundamental idea of the initial combination of enzyme and substrate.

With regard to the nature of this combination it seems more in accord with the available measurements to suppose that it is a chemical rather than a physical union. That it cannot be a matter of mere physical concentration of substrate molecules on the surfaces of the enzyme particles is evident in cases of the action of enzymes on such substances as proteins or starch, for in these cases the particles of enzyme and of substrate are probably of about the same size so that there would not be enough

room on the surface of the enzyme particle for any great concentration of substrate molecules. At the present time knowledge is being obtained as to the nature of this combination between enzyme and substrate by determining which groups in the *substrate* must be intact for the enzyme action to proceed. The active groups in the enzyme must then be such as are capable of combining with these essential ones in the substrate. For example, some proteolytic enzymes lose their power of acting on polypeptides if the terminal $\cdot\text{COOH}$ group of the polypeptide is esterified; evidently here it is the $\cdot\text{COOH}$ group that is concerned in the linkage. In other cases it has been shown to be the $\cdot\text{NH}_2$ group. But seeing that in each case a particular length and amino-acid constitution of the polypeptide chain are also necessary to render it vulnerable to the attack of the enzyme it is argued that the enzyme must combine with *two* points on the polypeptide chain. It is now well established that an enzyme may be capable of splitting a tripeptide of suitable constitution and yet be incapable of attacking the corresponding dipeptide containing one less amino-acid. This is evidently a matter of more exact adaptation than the mere possession of a peptide linkage for disruption.

Once the substrate molecule has combined with the enzyme it would seem that it comes under the influence of powerful electrostatic fields existing between the highly polar groups in the enzyme structure: the resulting stresses set up within the substrate molecule disturb the equilibrium of its constituent groups, so that the molecule either disrupts, or becomes "activated" in the sense that it now readily reacts with other substances such as, in the case of hydrolytic reactions, water. In order to express this activation by electrostatic stresses Haldane has modified

Fischer's lock and key simile for the relation of enzyme and substrate by saying that the enzyme key does not fit the substrate lock quite perfectly, but exercises a certain strain on it. With regard to the relation of these active groups to the enzyme structure as a whole there are two views. Some authorities would regard enzymes as definite chemical individuals, in which case the active part of the molecule might consist of certain groupings forming part of a long polypeptide or similar chain. On the other hand, others regard enzymes as complexes consisting of a small active group stabilised by a colloidal carrier and incapable of separate existence. We met a somewhat similar stabilisation of a small active labile group by a large inert protein molecule in the case of insulin. In support of this it has been found possible to transfer the active groups of some enzymes to other carriers than those with which they are usually associated in nature. Whichever of these views turns out to be correct, whether enzymes ultimately prove to be single chemical individuals or complexes of active groups and carriers it seems already certain that they are not necessarily proteins inasmuch as potent enzyme preparations *have* been made that do not give the colour reactions to which proteins respond.

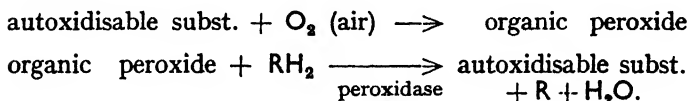
OXIDATION CATALYSTS

On account of their importance in accelerating the reactions that are the immediate sources of energy in the tissues, the enzyme systems concerned with oxidative processes are of special interest. It is hardly necessary to point out that the substances that are found to be rapidly oxidised in tissues are as a rule quite stable towards ordinary gaseous or dissolved oxygen alone. Glucose,

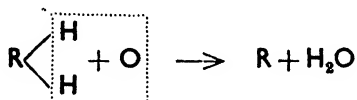
for example, is not attacked by oxygen even when left in contact with it for years. It is evident then that the tissues must possess powerful catalytic agents whereby such oxidations are accelerated to an extent commensurate with the energy expenditure of the organism. These oxidising catalysts were first studied in plant tissues, the starting point of the investigations being the simple observation that many otherwise stable substances will absorb oxygen from the air if a little of certain plant juices is added to their solutions. In order to demonstrate this it is most convenient to use as substrate some substance whose products of oxidation are coloured, hence the extended use of the resin called guaiacum in experiments of this kind. If a solution of this substance is shaken with air no effect is observed, but if now a little potato juice be added to the mixture the guaiacum is at once converted to conspicuous dark blue oxidation products. Evidently the potato juice contains a powerful oxidation catalyst or **oxidase**. That this is not just a simple enzyme is shown by the fact that it can be easily resolved into two components by means of alcohol. If potato juice is dropped into alcohol a precipitate is obtained which on separation and solution in water is found to be devoid of the power of transferring atmospheric oxygen to such substances as guaiacum. But it *will* transfer oxygen from hydrogen peroxide; it is therefore said to contain a **peroxidase**. The full explanation of the oxidising power of the plant juice is that it contains **autoxidisable substances** of a phenolic nature that are capable of combining directly with atmospheric oxygen to form organic peroxides from which oxygen is readily transferred to an oxidisable substrate by the peroxidase that the plant juice also contains. The autoxidisable

phenolic substances remain in solution when the plant juice is treated with alcohol; it is their conversion to organic peroxides that is held to account for the brown material produced when a cut surface of a potato or an apple is exposed to the air.

If we represent the substrate being oxidised by the formula RH_2 and imagine that the process of oxidation removes its two hydrogen atoms as water we can summarise the whole sequence of changes somewhat as follows:—



As the autoxidisable substance is recovered unchanged the net result of this roundabout process is the same as if the substrate had been oxidised directly thus:—

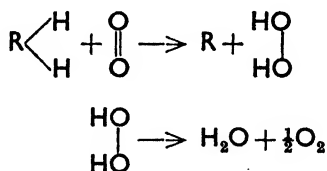


The thing is, however, that this direct action would not have occurred spontaneously; it requires the *activation of oxygen* by the peroxidase to bring it about. This discussion of the mechanism of oxidations brought about in the potato may seem rather remote from practical affairs, but actually it illustrates the principle employed in a very important test for blood, as, for example, in a specimen of urine. For the blood pigment hæmoglobin and its derivatives have the property of functioning as peroxidases. If, therefore, to the suspected fluid there be added hydrogen peroxide or ozonic ether (which contains ethyl peroxide) and either tincture of guaiacum, or

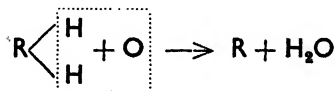
benzidine dissolved in glacial acetic acid, as oxidisable substrate the presence of even a trace of blood will reveal itself by the production of dark blue oxidation products.

In animals the oxidative mechanism differs fundamentally from that which we have just described in plants inasmuch as the enzymes found in animal tissues transfer or *activate* the *hydrogen* of the substrate instead of activating the oxygen that is to unite with it. We can perhaps best make this clear by a study of an animal oxidative enzyme that is found in milk. This is often referred to as the Schardinger enzyme. Out of a number of possible substrates for oxidation we will take formaldehyde; as hydrogen acceptor we will take not oxygen but methylene blue because it is converted into a colourless substance on reduction, so that its participation in the reaction can be readily followed. It is easy to show that in aqueous solution at body temperature formaldehyde does not react with methylene blue; but if both substances are added to milk and the mixture is kept warm the colour of the dye is soon discharged, showing that under the influence of the Schardinger enzyme the methylene blue has "accepted" hydrogen from the formaldehyde, which in turn may be said to have been oxidised by the dye. It is obvious that this reaction can be used for determining whether a sample of milk has been preserved by the addition of formaldehyde, or alternatively by being boiled, in which case, of course, the enzyme will have been destroyed. But its importance is by no means confined to this practical application; it has a much wider interest inasmuch as it seems to typify a process by which oxidations are commonly brought about in animal tissues. The main difference is that there, in place of methylene blue, we have as ultimate hydrogen acceptor the molecular oxygen

brought by the blood. When molecular oxygen acts as hydrogen acceptor it forms first hydrogen peroxide, and this substance has been shown to be produced, in traces at least, during the action of animal oxidising enzymes. Now hydrogen peroxide is a powerful tissue poison; it does not accumulate in any quantity, however, for all tissues where vigorous oxidations proceed are found to contain an enzyme known as **catalase** that promptly decomposes hydrogen peroxide into water and oxygen. The presence of catalase in a tissue extract can be shown by the rapid evolution of oxygen that occurs when it is mixed with hydrogen peroxide; in this way it can be demonstrated in a large number of materials ranging from aerobic bacteria to mammalian blood serum. Catalase differs from the plant peroxidases in that it acts only on hydrogen peroxide and not on peroxides in general. Representing the successive steps of these processes in the same way as before we have:—

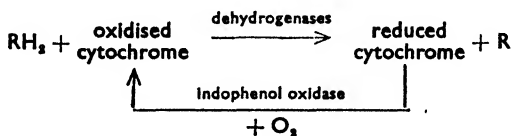


from which it will be seen that in effect the net result is the production of the same process of oxidation as we have been considering throughout:—



But we know now that a certain amount of the hydrogen activated in the combustible materials of the

cell (by **dehydrogenases** something like the Schardinger enzyme) is not directly accepted in this way by molecular oxygen, but goes to reduce **cytochrome**, an iron-containing pigment present in all cells where oxidations take place. This "cell colour" consists of a complex of substances similar in chemical constitution to hæmochromogen—a substance we are going to meet among the derivatives of the blood pigment (p. 322). Cytochrome can be seen by its absorption spectrum (which can be observed in cells by means of a suitable combination of microscope and spectroscope) to be capable of existing in both oxidised and reduced, i.e. in ferrous and ferric forms. When it has been reduced it can be reoxidised by means of atmospheric oxygen, but for this yet another enzyme, the so-called **indophenol oxidase**, also known to be present in cells, is necessary. The central position that cytochrome occupies in the oxidation mechanism of the cell can therefore be represented thus:



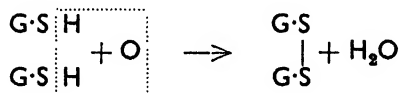
It is extremely interesting to find that, of the two types of agents that inhibit oxidations in tissues, anæsthetics such as chloroform act by preventing the reduction of cytochrome, while cyanides, carbon monoxide and sulphides act by preventing its reoxidation. We should mention that the indophenol oxidase that oxidises cytochrome is so named merely because it can catalyse the oxidation of a mixture of *p*-phenylenediamine and α -naphthol to "indophenol blue," and that the complex of cytochrome with this oxidase constitutes what has

previously been called the **Atmungsferment** or respiratory enzyme *par excellence*. It will be noticed that it is really only a part of the whole oxidative mechanism.

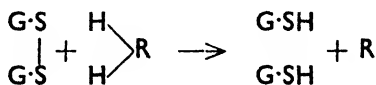
But cytochrome is by no means the only cell constituent that is capable of being alternately oxidised and reduced in animal tissues. From a number of different kinds of tissue there has been isolated a sulphur-containing substance called **glutathione**, which is now known to be a tri-peptide of the following structure:

glutamic acid—cystein—glycine.

Like cystein itself this glutathione, at all events in the presence of traces of iron, is readily oxidised in the air to a disulphide form corresponding to cystine (p. 19). Representing the main part of the molecule by **G** we have:



and this disulphide form is capable of acting as a hydrogen acceptor from oxidisable tissue constituents thus:



It will be seen that by this series of reactions we have again brought about the oxidation of RH_2 to R and H_2O , and undoubtedly glutathione *can* catalyse the oxidation of certain materials in this way, but the extent to which it does so in the tissues is still doubtful. One process in which glutathione seems to be directly concerned is the conversion of methylglyoxal to lactic acid (p. 194). It

here acts as a "co-ferment" to the enzyme glyoxalase. This suggests that even if glutathione does not directly catalyse tissue oxidations its sulphhydryl and disulphide forms (whose proportions will be determined by the prevailing oxidation-reduction potential) may to varying extents act as co-ferments to various tissue enzymes and so serve to correlate tissue reactions with the oxidising power of the cell.

Yet another factor concerned in cellular oxidation is what is known as the **yellow oxidation catalyst** first isolated a few years ago from yeast. This is a complex of a yellow heterocyclic nitrogen-containing pigment called **flavine** (p. 300) with phosphoric acid and protein. It appears to accept hydrogen from oxidisable substrates in much about the same way as we have described for methylene blue: in fact this yellow oxidation catalyst has been somewhat paradoxically referred to as "the methylene blue of the cell." To its activity is ascribed the residual oxidation that still takes place in some cells even when the cytochrome mechanism has been poisoned by cyanide.

Such, then, is the barest outline of the knowledge that has been won in recent years with regard to the oxidation catalysts in living matter. Even without further details it will be obvious to the reader how complicated the subject is. It is still under active investigation; but it will probably be some time yet before its complexities will have been so far unravelled as to make it possible for us to say exactly what part is played by each of the factors we have mentioned in the oxidation of any one of the substances that the living cell is known to burn, or to realise all that is implied when we say that a tissue obtains energy by the combustion of fuel to carbon dioxide and water.

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CHAPTER XV

ACCESSORY FOOD SUBSTANCES OR VITAMINS

"We came to few places where either the art of man or nature did not provide some sort of refreshment or other, either of the animal or vegetable kind. It was my first care to procure what could be met with of either by every means in my power, and to oblige our people to make use thereof, both by my example and authority; but the benefits arising from such refreshments became so obvious that I had little occasion to employ either the one or the other."—*Captain Cook*.

EVER since the time when shipping first became sufficiently developed to enable long voyages to be taken out of sight of land it has been a matter of common experience that a diet composed entirely of preserved foodstuffs is not adequate for maintaining the members of a crew in health over long periods of time, although the preserved food may contain sufficient fuel substances to supply the energy needs of the body, and suitable proteins for repairing its wear and tear. The commonest disorder that is liable to appear under these conditions is the disease known as **scurvy**, in which the chief characteristic is the occurrence of numerous small hæmorrhages throughout the body, more particularly between the teeth and round the bones and joints, the end effect being almost complete loss of muscular power in the limbs. At first these "scorbutic" symptoms were ascribed to the excess of salt used to preserve the food, but now it is realised that they are due to the absence from the preserved rations of small quantities of some substance that is essential for the maintenance of health, and that, though originally present in the fresh foods, is so readily decomposed as to be lost during the processes of preservation. That this is the correct explanation is shown by the fact that the missing substance can be supplied in sufficient quantity if small amounts of fresh fruit or vegetables are added to the dietary. It was known as long as a century ago that the juice of the lemon

(*Citrus medica* var. *limonum*) is particularly effective in preventing scurvy and that it retains its effectiveness on storage for a considerable time. But at that time it was thought that it was the acid of the lemon juice that was the potent constituent, and so there seemed no reason why the juice of the West Indian lime (*Citrus medica* var. *acida*), which is just as acid, should not be equally effective. It became the custom, therefore, as is well known, for expeditions to the arctic and other desolate regions to carry a supply of lime juice. But this often proved to be ineffective in preventing scurvy, and we now know that the active substance is not the citric acid of the juice, but some other substance which is not so abundant in lime juice as in lemon juice. Nowadays it is always concentrated *lemon* juice that is taken.

From this example it will be seen that a diet is not complete unless it contains, in addition to the requisite bulk of proteins, fats, carbohydrates and inorganic salts, small but adequate amounts of certain "accessory factors" found in fresh, i.e. uncooked, foods. It is these factors that are now generally known as **vitamins**, a name based on the now disproved supposition that these substances were *amines* necessary for the normal carrying on of *vital* processes. If the diet is deficient in these vitamins the deficiency shows itself by the onset of deficiency diseases of which scurvy is but one. This anti-scorbutic vitamin, whose action we have briefly described, is called vitamin C, because, although it was among the first whose action was discovered, it was the third to receive an alphabetical designation.

If we deal with the others in the order in which they were recognised we must go back to 1897, when Eijkman, the medical officer of a prison in Java, was investigating the disease beri-beri, which is common in the East among

natives whose staple article of diet is rice. This disease involves a widespread neuritis, a degeneration, that is, of all the peripheral nerve trunks so that loss of sensibility and muscular paralysis and degeneration result. Death by heart failure usually follows if the case is not treated. It became prevalent only when the natives gave up their crudely milled "paddy" rice and took to eating white polished rice, as we know it, with the outer reddish bran and the embryo "polished" off in modern steel mills. Eijkman happened to notice that on one occasion the poultry kept in his prison yard suddenly developed symptoms of beri-beri similar to those of his prisoners, and, on enquiry, found that the sick birds had been fed on the remains of the prisoners' diet of polished rice, instead of being allowed to wander round and find a mixed diet for themselves. This showed that the disease was caused by the diet, and after a period during which it was thought that it was due to the excess of starch, it became realised that it was really due to the *lack* of some substance present in the outer layers of the rice grain that are removed by the polishing. Then in 1911 Funk succeeded in obtaining from rice polishings by alcoholic extraction a material a small dose of which would cure a bird of beri-beri in a day. It was to this material that the name *vitamine* (then spelt with an "e") was first applied. It is now known as vitamin B, which is fortunate, as there is no difficulty in remembering that vitamin B is curative for beri-beri.

Meanwhile, Hopkins, as a result of experiments carried out between 1906 and 1912, had shown that for the processes of normal *growth* small amounts of substances other than the well-known requirements of proteins, fats, carbohydrates and inorganic salts were necessary in the diet. In his best known and most striking experiment

he took two batches of young rats and fed one batch on a mixture of carefully purified casein, starch, cane sugar, lard and inorganic salts, and the second batch on the same mixture with the addition of 3 c.c. of fresh milk per day per animal—an addition that, as Hopkins says, gave them hardly enough solid material “to nourish the tips of

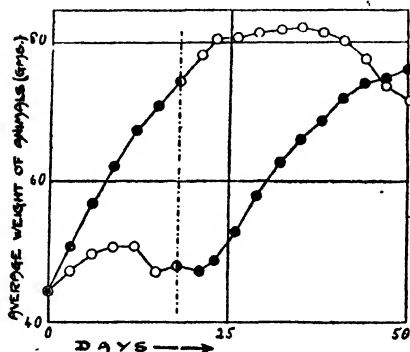


FIG. 13. To illustrate the influence of the vitamins in fresh milk on the growth of rats.

The group of animals whose average weight is represented by \circ received the basal diet only; those represented by \bullet received a little fresh milk per day as well. On the eighteenth day (represented by the dotted line) the diets of the two groups were interchanged. (From Hopkins.)

their tails.” The rats all ate with equal avidity, and yet those that received the basal diet only—without the milk—failed to put on weight, while those that received the milk grew normally. That this was not due to any undetected difference between the rats of the two batches was proved by interchanging the diets on the eighteenth day of the experiment; it was then found that those rats that previously had received no milk at once started growing as soon as it was given them, while those that had previously been growing put on no more weight, and even declined, when they were deprived of it (Fig. 13). There is no doubt now that in this striking result several dietetic factors must have been concerned. Of these the growth-promoting substance present in the fat of milk was the first to be recognised so it was called **fat-soluble A**

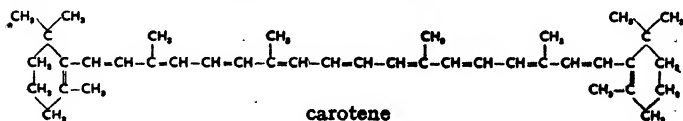
or more usually simply **vitamin A**. Not long again and it was found that fats of animal origin had the power of preventing the disease **rickets (rachitis)** in which the calcification of the bones and teeth is markedly deficient. It is only natural that at first this effect should be ascribed to the same fat-soluble vitamin as promotes growth, but when it was found that the anti-rachitic properties of a fat are not so readily destroyed by processes of oxidation that are sufficient to destroy its growth-promoting properties it was clear that the anti-rachitic vitamin is a different substance from vitamin A, so, the other intervening letters having been already assigned, it is named vitamin D.

In these various ways, then, the existence and importance of these four chief vitamins A, B, C, D came to be recognised. With this fundamental knowledge as a starting point there has been carried out during the last decade an enormous amount of patient and laborious work with the object of determining the exact physiological effects of these vitamins, their distribution in the multifarious foodstuffs that make up our diet, their stability during the processes of cooking, preserving and storing of foods, and of devising methods suitable for their concentration and isolation, with the ultimate goal of a determination of chemical structure and a possible synthesis always ahead. Patient and laborious this vitamin work has had to be because in general it is not easy to detect the presence of a vitamin or to judge of its amount by a simple, rapid test-tube reaction. It must usually be detected and estimated by its physiological effect, to observe which animal feeding experiments of many weeks' duration are often necessary. For the purpose of giving a survey of the achievements that have been made along these lines it will be convenient to deal

with the several vitamins in alphabetical order rather than in the order of their discovery in which we introduced them to the reader.

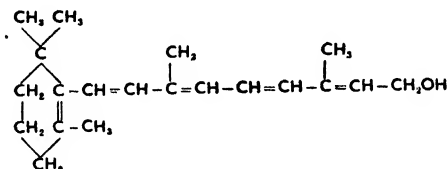
Vitamin A has been found most abundantly in the liver oils of fish—cod-liver oil, and especially halibut liver oil: in these it is accompanied by vitamin D. It is also abundant in the livers of the ordinary food animals (ox, sheep and pig) from which vitamin D is absent, and, as a further distinction from vitamin D, is moderately abundant in green vegetables. It not only promotes growth, but seems to increase the resistance of the body to bacterial infection. At all events it is definitely known that an infection of the conjunctiva and cornea of the eye leading to the condition known as **xerophthalmia** (dry eyes) can be produced in rats by an absence of this vitamin, and a war-time outbreak of the same disease among the children in Copenhagen (some of whom were blinded as a result) was definitely ascribed to the same deficiency.

Regarding the chemistry of vitamin A we already possess a considerable amount of information. It was early noticed that the vitamin A potency of plant materials runs parallel with their degree of pigmentation. Now the plant pigments that, like vitamin A, are "fat-soluble," are found to be complex hydrocarbons containing a long unsaturated carbon chain and often ring structures as well. These substances are exemplified by the hydrocarbon **carotene**, $C_{40}H_{56}$, which is the characteristic orange-coloured constituent of carrots and gives the name of **carotenoids** to the group. Its constitution is represented more picturesquely as follows:—



Now the important observation has been made that although vitamin A is not identical with carotene the hydrocarbon when fed to an animal is converted into the vitamin and stored in the liver (hence the importance of liver as a source of vitamin A).

It seems as if in the body the molecule of the hydrocarbon is split at the middle of the long carbon chain with the addition of a hydroxyl group so that vitamin A comes to contain one of the rings and half the unsaturated chain present in the original carotene, thus:—



According to this it possesses the same skeleton as a ketone called **ionone**, which is manufactured synthetically as a perfume on account of its violet-like odour (Gr. *ἰόν* = a violet), an odour which, significantly enough, vitamin A preparations also possess. If this is really so it should not be long before the vitamin itself is synthesised.

The main point in the recent history of vitamin B is the discovery of its complexity. Some time ago it was discovered that the rat requires for normal growth not only the fat-soluble vitamin A, but also a water-soluble factor that was at first supposed to be identical with the anti-beri-beri (anti-neuritic) vitamin B. But later it was found that the addition to the diet of maize (which is known to protect well against beri-beri) did not lead to a resumption of growth. Evidently then the water-soluble substance

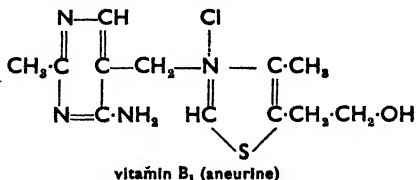
required for growth is not the same as that which prevents beri-beri. As the anti-beri-beri vitamin was the first to be described, this, therefore, received the symbol B_1 , and the growth-promoting factor was called B_2 . (In passing it might be pointed out that in Hopkins' experiments already described we now see that he was withholding from his rats both the fat-soluble A and the water-soluble B_2 , so that it is not surprising that he obtained so striking a retardation of growth.) But not only did the rats fail to grow on a maize diet deficient in B_2 : they also developed a condition of dermatitis at first thought to be identical with that observed in the human disease **pellagra** (It. *pelle agra* = "rough skin"), which is prevalent among the maize-eating peasant populations in Central Europe, and during the last two decades has appeared among the poorer classes of the population of the southern United States. The way in which Goldberger showed that this formidable disease, which produces not only skin lesions, but an inflammation of the whole of the alimentary canal accompanied by diarrhoea, and even nervous and mental disturbances, is due neither to an infection nor (as was later thought) to a deficiency of essential amino-acids in the diet, but to the lack of a water-soluble factor present in yeast, forms an interesting chapter in the history of medicine. This pellagra-preventing factor PP (or vitamin G as it is sometimes called after Goldberger) has now been proved not to be identical with the substance whose lack produces dermatitis in rats. This latter is now called B_6 , as meanwhile a further constituent of the vitamin B complex, B_4 , had been described as necessary for complete nutrition of the rat, while yet two others, B_3 and B_5 , had been found to be required for the normal growth of the pigeon. It will thus be seen that what was originally called vitamin B_2 is

a complex mixture of substances with a wide variety of actions.

By this time the reader will have realised that the one great difficulty in the investigation of these constituents of the vitamin B complex is that they all occur together. As we have mentioned, the richest sources of them are the bran and germ (embryo) of dry cereals, such as wheat and rice. Maize alone contains B_1 without " B_2 ." Yeast and yeast extract "marmite" contain them all abundantly, and so constitute a happy hunting ground for vitamin B chemists. Egg-white contains only " B_2 ," but the other member of the pair B_1 is in the yolk.

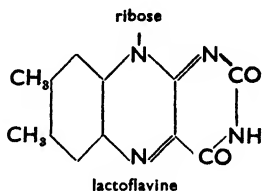
If we now enquire into the chemistry of all of these substances we find that, curiously enough, although the anti-beri-beri vitamin B_1 was the first claimed to have been isolated, it was one of the last to have its chemical constitution elucidated. Potent crystalline preparations of it were made from rice polishings and from yeast, and it was known to be a basic substance containing both nitrogen and sulphur, but it is only recently that it has been shown that this sulphur takes part with the nitrogen in forming a five-membered heterocyclic "thiazole" ring.

The other part of the molecule consists of the "pyrimidine" half of the purine ring (p. 109) thus:



and now that its chemical constitution is established vitamin B_1 has received the name **aneurine** in reference to its anti-neuritic function.

The growth factor of the vitamin B_2 complex turns out to be identical with a yellow water-soluble substance **lactoflavine** obtained from milk (Lat. *flavus* = yellow). It is akin to the flavine we just mentioned as a constituent of the yellow oxidation catalyst (p. 288). These flavines have been shown by synthesis to consist of a complex heterocyclic "isoalloxazine" ring system coupled up with a sugar (in this case ribose) and the *iso*-alloxazine itself is composed of a benzene ring united by two nitrogen atoms to what is virtually a pyrimidine ring again.



The pellagra preventing factor seems to be identical with a simple *pyridine* derivative—**nicotinic acid** (so called because it is a oxidation product of nicotine). Nicotinic acid and its amide will certainly cure pellagra.



And lastly, vitamin B_6 , the only other constituent of the complex to which we can assign a formula, has just been shown to be another simple pyridine derivative. On account of its function in preventing dermatitis it is now called **adermin**.



In contrast with all this, vitamin C, the anti-scorbutic factor, has retained its unity unassailed. On account of its importance its distribution has been very carefully studied, and it is now known to be present in greatest amounts in citrous fruits such as (to revive childhood's

memories) "oranges and lemons." From the latter the vitamin can be concentrated until the whole of the anti-scorbutic properties of a litre of the lemon juice may be contained in a single "acid drop" with a volume of about a cubic centimetre. The difference between this and the behaviour of the vitamin in lime juice on preservation has already been pointed out. Another valuable source of this vitamin is the tomato, which fortunately retains its anti-scorbutic properties on preservation provided that it be tinned whole. Then again the vitamin is abundant in green vegetables, cabbage, lettuce and water-cress, but as it is, of all the vitamins, the most easily decomposed on heating not much of it will be left in boiled-out cabbage, particularly if it be cooked with the addition of washing soda "to keep it a good colour." Speaking of cabbages reminds one that there is another common cruciferous plant, *Cochlearia officinalis*, whose anti-scorbutic properties have been so long known that it goes by the popular name of "scurvy-grass." Vitamin C is not present to any appreciable extent in dry seeds: since it is present in green leaves it must be synthesised by the seedlings during germination. On the basis of this consideration there has now been revived the hundred-and-fifty-years-old suggestion that one of the readiest ways of obtaining anti-scorbutic material in isolated situations would be to carry dry peas, beans, or grain, and to germinate these on the spot as required.

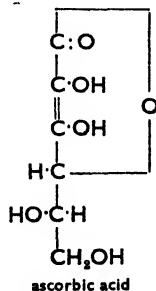
An interesting fact about scurvy is that all animals are not equally affected by its ravages. The rat, for example, which is used so extensively in other dietetic experiments, cannot be made to exhibit the symptoms of scurvy, no matter how deficient its diet; the guinea-pig has therefore been used practically exclusively as the subject of

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vitamin C research ever since 1912, when Holst, in Oslo, made the first systematic observations on the effects of depriving his pets of their greenstuff.

Since then, however, two decades had to elapse before vitamin C was definitely isolated and its nature determined. It turns out to be identical with an acid of formula $C_6H_8O_6$ previously found not only in lemons and cabbage, but also in the cortex of the suprarenal glands. At present its most abundant source is the red pepper that is a common article of diet in central Europe. The acid itself is probably an oxidation product of a hexose sugar; it possesses a labile hydrogen atom whereby it can exist in an oxidised and a reduced form, and so possibly play a part in the catalysis of tissue oxidations. At the same time it will effectively protect guinea-pigs against scurvy. To express this latter property this remarkable substance is now named **ascorbic acid**.

Recent advances in the study of this vitamin have included not only the determination of its constitution but its synthesis using the sugar galactose as a starting point. From its formula it will be seen that although it is an acid capable of forming a sodium salt it possesses no carboxyl group, i.e. no $\cdot OH$ group attached directly to a $:CO$ group.



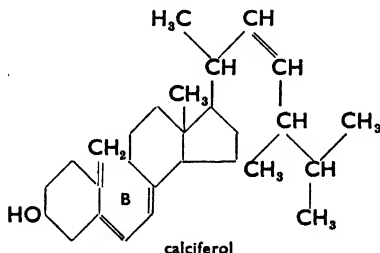
But it possesses the next best thing, namely an $\cdot OH$ group attached to a carbon atom adjacent to a $:CO$ group and thereby endowed with feebly acidic properties.

During recent years there has been a disgraceful abundance of opportunity for the study of vitamin D and the effects of its absence. For example, as a result of food shortage during and after the so-called "great" war,

rickets was rife among the child population of Vienna, while an incapacitating and painful softening and deformation of the joints (**osteomalacia**) affected a considerable proportion of the adults. It was therefore a matter of no great difficulty for the British and American relief workers there to prove conclusively that these conditions were not at all the result of bacterial infection, but were deficiency diseases arising as a direct result of the lack of anti-rachitic vitamin. They were treated by the administration of cod-liver oil, which is the richest readily accessible source of it. Years before this, however, there had grown up the firm conviction that rickets was in some way caused by lack of sunshine, and certainly it is of most frequent occurrence in town dwellers living in dark and dismal surroundings. The workers in Vienna took the opportunity of investigating this question and proved abundantly that exposure to sunlight does prevent or even cure rickets. Later experiments on dogs fully confirmed this result and demonstrated quite clearly that rickets does not occur in animals exposed to sunlight even when they are receiving a diet on which similar animals kept in the dark do develop the disease. Then it was discovered that it was sufficient to prevent rickets if merely the food and not the animal were exposed to light, from which it at once became apparent that the action of the light is to form vitamin D from some precursor present both in the constituents of the diet and in the tissues of the animal. Naturally the next step was to determine what this vitamin precursor was; it was soon identified as a sterol and was at first thought to be cholesterol, but ultimately was shown by its absorption spectrum to be **ergosterol** (p. 146).

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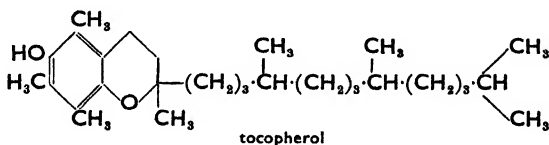
Nowadays the process of irradiating ergosterol has been much perfected; its solution in ether is circulated for several hours through a quartz tube exposed to the radiation from a mercury arc lamp. It is thereby converted into a mixture of substances from which by suitable chemical means vitamin D can be separated. This synthetic anti-rachitic vitamin is so potent that one part of it can lead to the deposition of some three million parts of calcium phosphate in the skeleton of an animal! — from which property it has received the name **calciferol**. It turns out to be isomeric with the ergosterol from which it is obtained, so the effect of the light is evidently to produce an intra-molecular rearrangement, most probably an opening up of ring B as shown in the formula:



In actual fact the vitamin as it occurs in cod-liver oil seems to be not quite identical with this calciferol, but to differ from it only in possessing the side chain of cholesterol instead of that of ergosterol. Just how these substances facilitate the absorption and transport of calcium in the organism is a fascinating problem awaiting further investigation.

For the sake of completeness we should mention a further fat-soluble vitamin — E. This is a substance found to be necessary for reproductive fertility of rats

of both sexes. It is present in many animal and vegetable foodstuffs, but occurs (being fat-soluble) in greatest concentration in the oils obtained from seeds and other vegetable tissues. Unlike most substances concerned with sex it is not a sterol, but possesses a heterocyclic oxygen-containing ring system, called chromane because of its occurrence in flower pigments, united to a side chain similar in structure to phytol, which is a component of chlorophyll. The modern name of this vitamin is **tocopherol** (Gr. *τόκος* = birth). Very little is known at present as to the relation between vitamin E and human reproduction.



In concluding this account of the vitamins we would refer once again to the relation of these substances to human dietaries. From what we have already said, the reader will have abundantly realised that the vitamins represent much more than mere attractive problems in organic chemistry. They present us with the means of attaining an ideal—the ideal, namely, of wiping out completely all these entirely preventable deficiency diseases. From our modern knowledge of the synthesis of vitamin D those who are concerned with the much neglected problem of the cleaning of the wickedly and unnecessarily polluted atmospheres of our large towns will receive much encouragement and justification for their efforts. Then again it cannot be borne too clearly in mind that the need for vitamins exists not only acutely

on long sea voyages, arctic expeditions, or in beleaguered cities, but continuously among all sections of an ordinary peaceable workaday population. Of course a well-nourished individual who has means enough to choose for himself a "good mixed diet" will not suffer from rickets, scurvy or beri-beri. But it is quite otherwise for infants, or for members of institutions; they are at the mercy of those who provide for them. It is of the utmost importance, therefore, that those who are in this way responsible for the feeding of others should be well acquainted with such circumstances as the variability of milk as a source of vitamins. They should realise, for example, that winter milk from cows confined to their stalls and fed on vegetable oil-cake and hay is not likely to contain much of either vitamins C or D, and that what it does contain will be still further reduced if the milk is sterilised by heat or converted into powder by drying. Infants fed on it may therefore develop rickets, with its lack not only of skeletal, but also of mental development, or incipient scurvy with its tenderness of gums and painfulness of joints and general irritability. Hence the importance of supplementing the daily rations of an infant with a little cod-liver oil and the juice of an orange. Hence also the value of buying, during our winter, butter that has been produced in the sunshine of the southern hemisphere. But it must not be supposed that it is always those in authority who err. Even folk providing for themselves have been known to take so little heed of the exhortation to "eat more fruit" as to suffer from scurvy — particularly is this the case among elderly bachelors or spinsters living alone. The moral of which, as of much else that we have said in this chapter, should be obvious.

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CHAPTER XVI

PROTECTIVE SYNTHESIS: DETOXICATION

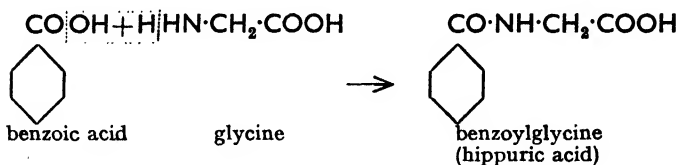
"... events which ultimately result in the modification of the disturbing substance and its extrusion from the tissues concerned in a form less noxious to the body as a whole."

—*Hopkins.*

THERE are several ways in which the body can overcome the harmful effects of poisonous substances that are taken into it or are produced during the course of metabolism or bacterial action in the tissues. The formation of what are called anti-bodies, which combine with and neutralise the poisons or toxins liberated during the development of bacteria, is nowadays a process so familiar as to form a piece of everyday knowledge. A less generally realised but very important further method which the body possesses for combating poisons is that known as protective synthesis. This is a process whereby a poisonous substance of comparatively simple chemical structure is built up in the body into a more complex product by which the poisonous properties of the original harmful substance are no longer exhibited, so that the poison is masked until it can be eliminated from the body. To take an example—an animal is given a dose of benzoic acid. This is a poison that cannot be oxidised away in the tissues, so that it would tend to remain where it was carried and to exercise its poisonous action. But it is found that the benzoic acid does not long remain as such; it is very soon built up with glycine into a more complicated substance known as **hippuric acid**, which is a comparatively harmless substance, and is excreted by the

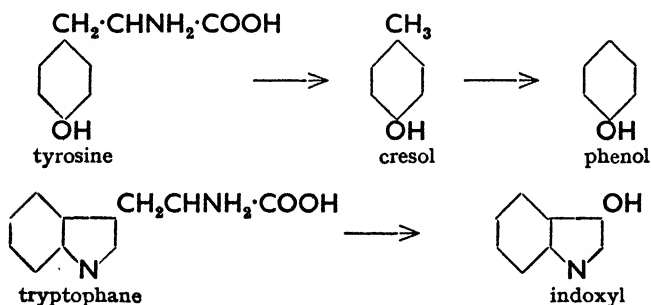
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kidney. Hippuric acid is benzoylglycine: its formation can be represented as follows:—



That is a typical example of what we mean by protective synthesis. The body defends itself against the benzoic acid by synthesising from it a more complicated but less harmful product. It is not surprising that it should be glycine which is used in this way by the body, for there is always an abundance of glycine in the tissues; it is usually present in large amounts in the food, and even when it is not supplied the body can synthesise it (see page 103). Benzoylglycine is called hippuric acid because it occurs largely in the urine of the horse and other herbivorous animals. An animal that feeds mostly on vegetable food takes into its body with its diet quite a large amount of benzoic acid, and therefore uses this protective mechanism to a larger extent than an animal that eats little plant food. Thus we find more hippuric acid in the urine of a horse than in that of a man. This formation of hippuric acid furnishes the body with one method of protection against harmful ring compounds. But there are others. It is well known that in the alimentary canal bacteria abound. Now among the reactions brought about by intestinal bacteria the most important, from our immediate point of view, are the attacks on the molecules of tyrosine and tryptophane. The bacteria, as one may put it, "bite off" portions of the side chain of these amino-acids, and then the residual substances still containing

the ring intact are no longer capable of being oxidised in the body. We have already mentioned that tyrosine and tryptophane are exceptional among ring compounds in the ease with which they are completely oxidised by the body. A comparatively small change in the structure of the side-chain is sufficient, however, to prevent the ring from being oxidised. The result of the bacterial action in the alimentary canal is the formation of such substances as **phenol** and **cresol** from tyrosine and **indoxyl** from tryptophane. The way in which these products arise will be clear from a study of the formulæ. Note that in each case the ring remains but that the side chain has been attacked:—

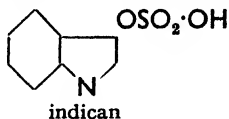


Now these phenols are too poisonous to be allowed to pass into the general circulation. But when these substances are absorbed from the alimentary canal, on reaching the liver they are coupled up with sulphuric acid, forming what are known as **ethereal sulphates**. The sulphuric acid forms an ester with the hydroxyl group and so masks the poisonous properties this group possesses in these compounds. These ethereal sulphates of phenol, cresol and indoxyl are therefore much less poisonous than

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the original substances themselves; they can be carried round the circulation without harm until they arrive at the kidney to be excreted in the urine.

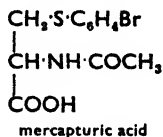
Of these ethereal sulphates, indoxyl sulphate — which is otherwise known as **indican** — is of particular importance because it can be easily tested for. All that is necessary is to add to 10 c.c. of urine rather more than an equal volume of strong



hydrochloric acid and one, or at the most two, drops of 2 per cent. potassium chlorate solution. The indican is thereby oxidised to indigo blue itself, and this can be dissolved out and made more evident by shaking the reaction mixture with a few drops of chloroform. Normal urine gives only a very faint tinge of blue colour in this way, and anything more than this indicates an abnormally large production of ethereal sulphates such as occurs as the result of excessive bacterial breakdown of proteins either in the alimentary canal during constipation, or, possibly, in a large abscess. Such a rough diagnosis can be confirmed by the quantitative estimation of the total ethereal sulphate of the urine, which is carried out by determining first of all the inorganic sulphate and then taking another sample of the urine, boiling with dilute hydrochloric acid to hydrolyse the ethereal sulphates, and then estimating the total sulphate, as before. This now includes both the sulphate of the inorganic sulphates and also that obtained from the ethereal sulphates. The inorganic sulphate having been already determined, the amount of sulphuric acid combined as ethereal sulphate is known.

In dealing so far with the formation of hippuric acid and ethereal sulphates we have been considering processes

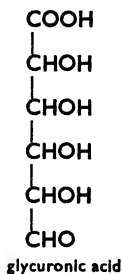
that occur to a certain extent even in the normal body. But it is very interesting also to consider the behaviour of the body to substances entirely foreign to itself—to substances with which it cannot possibly have been in contact during its evolutionary history. In this way we not only gain an idea of the synthetic resources available to protoplasm for meeting chemical situations that are entirely new to it, but we also obtain information of very practical importance, because many such "foreign" substances are administered to the body as drugs, and it is essential to know the forms in which they may be excreted in the urine in order to be able to interpret with certainty the results of urine analyses. Let us take, for example, such a substance as brombenzene— C_6H_5Br . The student of organic chemistry knows that this is a very stable inert substance, a halogen atom attached to a ring not displaying the characteristic reactivity that we usually associate with such atoms. Nor is the bromine separated off by living tissues, but nevertheless the body can bring the brombenzene to react with the amino-acid cystein (p. 20) with the formation of bromphenyl cystein, which, in the form of its acetyl derivative, known as **mercapturic acid**, is excreted as part of the "neutral sulphur" fraction of the urine. A part of the brombenzene is also excreted as ethereal sulphate which indicates that the body must have succeeded in inserting an $\cdot OH$ group into the ring. And even benzene itself, in spite of all we usually say about the stability of rings, forms *some* ethereal sulphate in the body, which means that it must first have been converted into phenol—a change that in the laboratory requires first treatment of



the benzene with fuming sulphuric acid and then fusion with potash of the resulting sulphonic acid!

Yet another method of dealing with foreign substances is to couple them up with **glycuronic acid**, a substance that is derived from glucose by the oxidation of the terminal $\cdot\text{CH}_2\text{OH}$ group to a $\cdot\text{COOH}$ group:—

This occurs not only as an alternative method of eliminating phenol (which may enter the body from a carelessly dressed wound) but also with very many of the drugs that are intentionally administered — chloroform, chloral, morphine, antipyrine and so on. Now in these glycuronates the oxidisable $\cdot\text{CHO}$ group of the original glycuronic acid is not in any way masked, so that these substances reduce Fehling's solution and so might give rise to an erroneous impression that sugar was present. The moral of which is that one must not attribute a reduction of Fehling's solution by a sample of urine to sugar without taking into account the drugs with which the patient has been treated!



In addition to all of this the body has been shown to be able in some cases to reduce $\cdot\text{NO}_2$ to $\cdot\text{NH}_2$ groups, to acetylate amino-groups and even to introduce $\cdot\text{CH}_3$ groups into rings as, for example, in the case of pyridine (p. 83). To describe these matters in detail would, however, take us far beyond our scope, but we have probably said enough to give the reader a glimpse into this fascinating field.

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A more popular account, which all students should read, is given in Hopkins' Presidential Address to the Physiology Section of the British Association for the Advancement of Science at its meeting at Birmingham, 1913. Its title is

"*The Dynamic Side of Biochemistry*,"

and it will be found in the British Association Report for that year.

CHAPTER XVII

THE PIGMENTS OF THE BODY

“Is it not strange to find this stern and strong metal mingled so delicately in our human life, that we cannot even blush without its help?”—*Ruskin*.

THE pigments of the body form a group of complex substances whose reactions will be of interest to anyone who has an eye for colour and a taste for rather complicated chemistry. By far the most important of the pigments occurring in the animal body is **hæmoglobin** — the scarlet colouring matter of the red blood corpuscles. It owes its importance to three chief circumstances. In the first place, it plays so essential a part in the carriage of the respiratory gases, oxygen and carbon dioxide; secondly, it gives rise during its decomposition in the body to the pigments of bile and fæces, and to one of the pigments of the urine; and, thirdly and lastly, it gives rise to a number of derivatives and related products that are of interest not only from the strictly chemical point of view, but also because they occur abnormally in the body under the influence of poisons or disordered metabolic processes. We shall deal with each of these topics, but not in the order in which we have mentioned them. It will be more convenient to begin with a consideration of the chemical relationships of hæmoglobin and its derivatives, and then to consider the metabolic changes that these substances undergo in the body. We shall reserve the question of the carriage of the blood gases for the next chapter.

To begin, then, with a consideration of the chemistry of hæmoglobin itself. The first fact to be noticed is that this substance is a conjugated protein, that is, that its molecule consists of a compound of a protein, **globin**, with a non-protein moiety now called **hæm**. Globin is a coagulable protein insoluble in water but soluble in salt solutions and characterised by a high content of histidine. Hæm is a compound of iron with a complex organic substance known as **proto-porphyrin**, whose constitution we shall discuss in a moment.

Hæmoglobin is exceptional among proteins in the ease with which it can be crystallised. In the case of the blood of some animals—notably that of the horse—it is necessary only to centrifuge off the red corpuscles, and then to disrupt them in some way, say, by treatment with a little ether, in order to liberate the hæmoglobin and so obtain a solution from which the pigment will crystallise out slowly when it is kept in a cool place. In other cases a little alcohol must be added in order that the hæmoglobin shall come out of solution.

The most characteristic property of hæmoglobin is its power of combining with oxygen to form the easily dissociable compound **oxyhæmoglobin**. This is, in fact, the product obtained by the method of preparation just given, for the hæmoglobin, if not completely saturated with oxygen in the original blood, will become so on exposure to air during the manipulations. The oxygen may be removed from oxyhæmoglobin in order to form hæmoglobin itself—"reduced" hæmoglobin it is often called—by exposure to a vacuum or to an atmosphere devoid of oxygen, or by treating the oxygenated substance with chemical reducing agents. Nowadays the modern reducing agent sodium hydrosulphite, $\text{Na}_2\text{O}_2\text{S}_4$,

is usually used for the purpose. The reduced hæmoglobin is of a purplish colour; on shaking with air it rapidly reabsorbs oxygen and becomes converted into the scarlet oxyhæmoglobin once more. As it is by means of their colours that these pigments are distinguished one from the other, it will be useful at this point to give an outline of the way in which this property is utilised for the purpose. That these substances possess colour at all depends upon the fact that, when white light passes through their solutions, some of the coloured rays, of which the white light is known to be composed, are absorbed, while the remaining coloured rays are relatively unaffected, and so, emerging from the solution, are visible to the eye. Or, if the white light is first spread out by means of a prism into a spectrum, so that its constituent rays of various colours, that is, of various wave-lengths, are all arranged in order, then, on viewing the spectrum through a layer of a particular pigment, this substance will absorb rays of certain particular wave-lengths so that only the remaining rays will get through. We shall see, therefore, only certain parts of the coloured spectrum; the remaining portions, where the corresponding rays have been absorbed by the pigment, appearing as dark **absorption bands**. The part of the spectrum which is transmitted through the solution is known as its **absorption spectrum**, and, seeing that each pigment possesses its own characteristic absorption spectrum, the spectroscope furnishes the readiest means by which these substances can be identified. Actually in practice it is usual to allow the white light to pass first through the solution to be examined and then by means of the prism of a spectroscope to analyse the mixture of rays which passes through in order to determine which wave-lengths have been

ABSORPTION SPECTRA OF BLOOD PIGMENTS

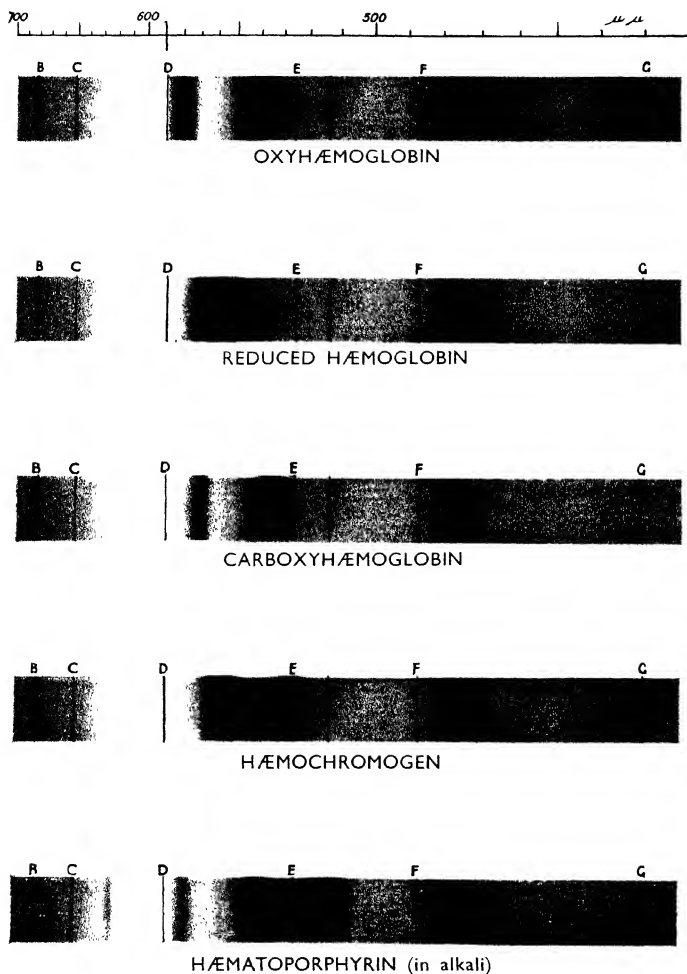


FIG. 14.

T.K.P.
ad nat. del.

absorbed. If we observe carefully the spectrum formed by sunlight we find that it itself is not continuous, but is crossed by a number of fine black lines known after their discoverer as Fraunhofer lines. These are nothing more nor less than the very narrow absorption bands of substances that exist in the form of vapour in the atmosphere of the sun, and which absorb certain rays from the white light sent out from the glowing interior. One of the most conspicuous of these lines is that labelled D, which can easily be shown to be an absorption band of sodium vapour. This occurs at a certain wave-length whose value has been determined by physical methods to be 589 millionths of a millimetre ($589\text{ }m\mu$).^{*} The wave-lengths of the other easily visible Fraunhofer lines have also been determined, and by means of these it is possible to construct a scale of wave-lengths so that the position in the spectrum of any absorption band given by a pigment can be accurately defined. It is to be noted that this scale of wave-lengths will not possess divisions of equal size because a prism spreads out the rays at the violet end of the spectrum to a greater extent than those at the red end.

To return now to the properties of the particular pigments we are studying—if we observe the absorption spectrum of oxyhæmoglobin—provided that the solution is not so strong as to block out the greater bulk of the spectrum altogether—it is easy to see that light is absorbed most strongly in two regions fairly close together in the green. Oxyhæmoglobin has, therefore, a double-banded absorption spectrum.

On the other hand, reduced hæmoglobin shows only a single absorption band which occupies the space between the inner edges of the two bands of oxyhæmoglobin,

^{*} Often—less correctly—designated $\mu\mu$.

as is seen in the spectra represented on the previous page.

As we have already said, a most characteristic property of hæmoglobin is its power to combine with gases. Next in importance to its compound with oxygen is that which it forms with carbon monoxide. Indeed, hæmoglobin combines more readily with carbon monoxide to form **carboxyhæmoglobin** (better but less often called **carbonylhæmoglobin**) than with oxygen to form oxyhæmoglobin; in fact if hæmoglobin be exposed to a mixture containing as little as one part of carbon monoxide to 300 parts of oxygen, about as much of it will be found to be combined with the one gas as with the other. A considerable percentage of carbon monoxide is contained in coal gas, so that if this be bubbled through diluted blood, carboxyhæmoglobin is readily formed. This carboxy-compound differs in several respects from oxyhæmoglobin. Its solution is of a bluish-red colour and not a full scarlet. Its absorption spectrum shows two bands, in the green, but these are slightly nearer the violet end of the spectrum than those of oxyhæmoglobin, as may be seen from a careful comparison of the two spectra as given in the figure. A simple test by means of which a solution of oxyhæmoglobin can be distinguished from one of the carboxy-compound is to dilute each with water until the colour is faint. It is found that in extreme dilution the oxyhæmoglobin appears more yellow than red, while the carboxyhæmoglobin remains bluish-red until the dilution has been carried so far that colour can no longer be detected. Chemically, carboxyhæmoglobin is distinguished from oxyhæmoglobin by the fact that it cannot be reduced to hæmoglobin by hydrosulphite or other reducing agents. It is of course

the potency of carbon monoxide in displacing oxygen from oxyhæmoglobin that accounts for the poisonous properties of coal gas, motor car exhaust gases, the "after damp" of coal mines, and other such atmospheres.

Before leaving the immediate derivatives of hæmoglobin we must mention that when oxyhæmoglobin is treated with an oxidising agent—potassium ferricyanide is the one usually employed—the whole of its oxygen is liberated as gas. The reduced hæmoglobin so formed is however at once reoxidised by the ferricyanide, not with the re-formation of oxyhæmoglobin, but with the production of a brown substance called **met-hæmoglobin**. This is an important reaction, because by collecting and measuring the amount of oxygen evolved the amount of oxygen present as oxyhæmoglobin in any solution can be estimated. Met-hæmoglobin will not give off oxygen when exposed to a vacuum, but it can be reduced to hæmoglobin by *chemical* reducing agents such as hydro-sulphite. It used to be thought to be isomeric with oxyhæmoglobin, differing from this latter merely in the firmness with which it held its oxygen. But several lines of recent research have indicated that it is a compound of hæmoglobin with only one atom of oxygen, whereas oxyhæmoglobin is a compound with two atoms which are, moreover, but loosely combined. Still more recently electrochemical studies have shown that the most important difference between met-hæmoglobin and oxyhæmoglobin lies not so much in their relative oxygen contents as in the circumstances that in met-hæmoglobin the iron is in the *ferric* (tri-valent) condition, while in oxy-hæmoglobin and in hæmoglobin itself the iron is in the *ferrous* (di-valent) condition. From this it follows that the combination of hæmoglobin with oxygen does

not, as might have been supposed, lead to the conversion of its iron from the ferrous to the ferric form. Met-hæmoglobin has a conspicuous absorption band in the red, but there are also two faint ones in the green and a general absorption of the blue rays. It is interesting to note that it can be produced by the action of a large variety of substances on hæmoglobin; this explains its occurrence in the blood of chemical workers who live in atmospheres containing nitrous fumes, or the vapour of nitrobenzene.

We have now dealt with the more important immediate derivatives of hæmoglobin in which the essential constitution of the molecule has remained undisturbed. We now pass on to consider the substances obtained by breaking down the original complex molecule to varying degrees.

When a solution of oxyhæmoglobin is treated with a few drops of caustic soda solution and warmed a brown alcohol-soluble pigment **hæmatin** is produced. At one time it was thought that the action of the alkali was to sever the union between the globin and the non-protein portion of the molecule so that hæmatin was regarded as being the free porphyrin-iron complex of the hæmoglobin molecule; but, as we are about to see, this idea has been disproved. If we treat reduced hæmoglobin with alkali—or alternatively treat oxyhæmoglobin with a reducing agent and alkali together—we obtain “reduced alkaline hæmatin,” which is also called **hæmochromogen**. This is a pink pigment showing two narrow absorption bands in the green, one of which is so much darker than the other and is so sharply defined and distinct (see Fig. 14) that the formation of hæmochromogen is used as a test for blood in urine, and in stains on such things as clothes.

weapons, etc. The stain is extracted with dilute caustic soda solution, a little hydrosulphite is added, and the mixture warmed to about body temperature: if the suspected pigment is actually blood the solution turns pink and shows the characteristic definite absorption spectrum of hæmochromogen.

Another test for blood that gives rise to a product of theoretical importance is the preparation of **hæmin** crystals. If a trace of blood or the scrapings of a blood-stain be just dried (without charring) on a microscope slide, and a drop of glacial acetic acid and a tiny crystal of sodium or potassium chloride be added, and the mixture, covered with a cover-slip, be then just brought to the boil, the liquid on cooling deposits a plentiful crop of small brown rhombic crystals of hæmin, which is known for certain to be the hydrochloride of hæm and therefore to be globin-free. Now if, as at one time supposed, hæmatin were really the protein-free iron-porphyrin complex of the hæmoglobin molecule and hæmochromogen the same complex in the reduced condition, it should be possible quite easily to convert hæmin into hæmochromogen by the addition of soda or potash and a reducing agent. But this has been found to be impossible: hæmin cannot be converted into hæmochromogen by the addition of alkali and reducing agent alone; there must also be added a protein or amino-acid or some other nitrogenous substance that will take the place of the protein. In the ordinary method of making hæmatin and hæmochromogen from hæmoglobin the protein globin is the nitrogenous substance present, and we conclude from this that hæmatin and hæmochromogen still contain the globin as an integral part of their molecules: it is not split off and left free in the

solution when hæmoglobin is treated with an alkali. The reason why for a long time this was not realised was that earlier workers had all used ammonia as the alkali in the conversion of hæmin into hæmochromogen, and, as it happens, even so simple a nitrogen derivative as ammonia can take the place of globin and produce an "ammonia hæmochromogen" whose spectrum was mistaken for that of true "globin hæmochromogen." Even such substances as nicotine and pyridine can form their corresponding hæmochromogens—a fact that is utilised in Takayama's test for blood, in which the material dried on a slide is treated in the cold with a mixture of pyridine, caustic soda and, as reducing agent, glucose. With this reagent hæmoglobin readily yields an abundance of characteristic pink crystals of "pyridine hæmochromogen."

The question now arises as to what is the nature of the difference between hæmoglobin on the one hand, and hæmatin and hæmochromogen on the other, if, as we have seen, each of these substances is a compound of globin and hæm. The modern answer to this question is that in hæmoglobin the globin is "*native*"—that is it has not been influenced by any of the reagents of a modern civilised laboratory, whereas in the derived products the globin has been altered, in some way not very clearly understood—"denatured," as it is said—by the alkali used in their preparation. We thus conclude that hæmoglobin itself is a compound of

ferrous hæm + native globin,

while hæmochromogen is a compound of

ferrous hæm + denatured globin.

As we have mentioned, when *hæmoglobin* is exposed to

atmospheric air two atoms of oxygen are taken up for each atom of iron in the pigment, but the iron is not thereby oxidised to the ferric condition. In order to bring about this oxidation a stronger oxidising agent than free oxygen is required. We have used ferricyanide, which then gives us **met-hæmoglobin**, the compound of

ferric hæm + native globin.

When the iron has been thus oxidised to the ferric condition the compound can no longer retain its hold on the two loosely-combined atoms of oxygen, which are therefore evolved. On the other hand when a more or less neutral solution of *hæmochromogen* is exposed to the air its iron is oxidised to the ferric condition by the free oxygen and a substance known as **parahæmatin** or **kat-hæmoglobin** results. As would be expected this compound of

ferric hæm + denatured globin

can also be prepared by the action of denaturing agents on met-hæmoglobin.

From this it will be seen that the globin of hæmoglobin exerts a subtle influence on the behaviour of the hæm with which it is combined, preventing it from being oxidised to the ferric condition by free oxygen, and so enabling it to combine loosely with the gas, but that this influence is lost when, as in hæmochromogen, the globin is denatured.

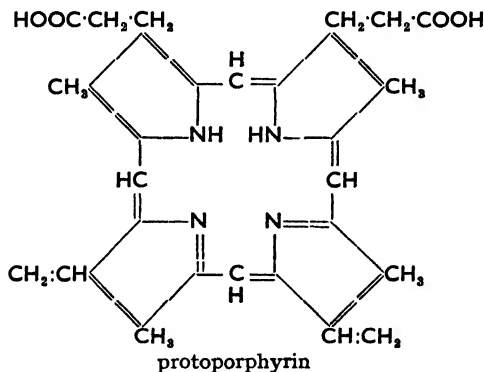
The brown substance hæmatin that we mentioned as an intermediate stage in the preparation of hæmochromogen from oxyhæmoglobin seems to be nothing more than an indefinite colloidal complex of hæm, oxidised to the ferric condition by the oxygen present, and globin

denatured by the alkali, the excess of which prevents the strictly chemical union of these components.

Having arrived at this point in our discussion we ought perhaps to mention that the same unfortunate kind of confusion of nomenclature exists in this as in so many branches of scientific study. In the account we have just given we have followed what would seem to be the most satisfactory way of naming the various substances concerned, but the student should be warned that ferrous hæm is by some authors called "reduced hæmatin," the name "hæmatin" itself being then given to what we have called "ferric hæm."

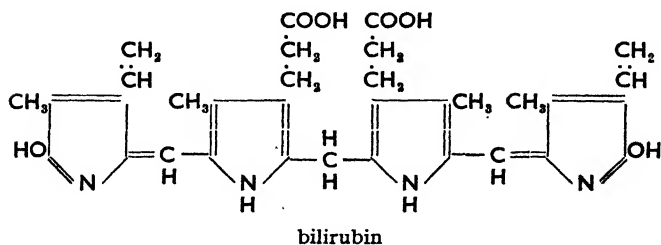
Returning, however, to our account of these pigments — when hæmoglobin is treated with concentrated acid, not only are the hæm and the globin separated, but the hæm is robbed of its iron, and the purple iron-free pigment **hæmatoporphyrin** remains (Gr. *πορφύρεος* = purple). The decomposition can be easily illustrated by allowing a few drops of blood to fall into strong sulphuric acid, when a purple solution containing hæmatoporphyrin is at once obtained. The substance itself can be precipitated by pouring the acid mixture into water and if this precipitate be collected and warmed with dilute alcohol containing caustic soda the resulting alkaline solution of hæmatoporphyrin shows a very characteristic absorption spectrum containing four bands (Fig. 14). This hæmatoporphyrin is but one example of the widely-distributed group of pigments known generally as the porphyrins in the molecules of which the essential structure is a complex of four pyrrole rings (refer back to p. 7 for the pyrrole ring). Actually as we stated on p. 317 the porphyrin present in hæmoglobin and its derivatives is **proto-porphyrin** and not this hæmatoporphyrin we have just

made; but the difference arises merely because, unless special precautions are taken, the protoporphyrin as soon as it is liberated combines at the double bonds of its $-\text{CH}:\text{CH}_2$ (vinyl) groups with two molecules of water, which after all do not make much difference in a molecule like this



Such porphyrins have been much studied of late, from the purely chemical and also from the biological point of view, and it has been realised that they and their derivatives are very widespread constituents of living matter. Not only hæmoglobin but many other animal pigments are porphyrin derivatives, as are also cytochrome (p. 286) and several other of the oxidation catalysts of the cell. So also is **chlorophyll** which is responsible for the photo-synthetic activities of green plants. It seems as if during the course of evolution the same piece of chemical structure has been utilised for a variety of very different functions. A further interest of these iron-free porphyrins from the point of view of human physiology is that they are closely related to, and indeed give rise to, the pigments of the bile. The average life of a red blood corpuscle

seems to be of only a few weeks duration. When its period of usefulness is at an end it is engulfed by one of a scattered collection of cells that together constitute what is known as the **reticulo-endothelial system**. The cells constituting this system occur in the reticulum of the spleen, and lymph glands, in the liver, the bone marrow and several other organs. In the reticulo-endothelial cells the hæmoglobin liberated by the disintegration of the effete red corpuscles is decomposed, and the resulting porphyrin is converted into the reddish bile pigment **bilirubin**. This and the liberated iron then find their way as normal constituents of the blood stream to the liver where the iron is stored until required for the formation of new hæmoglobin while the bilirubin together with that formed in the liver itself is excreted in the bile. Bilirubin possesses one less carbon atom than proto- or hæmatoporphyrin. During its formation in the body one of the :CH links is removed from the original porphyrin structure, the point of rupture being marked by two ·OH groups, so that the four pyrrole rings may be regarded as forming a straight chain instead of a "porphine" ring:



The decomposition of hæmoglobin in the body therefore proceeds further than it does when this substance is acted upon by strong sulphuric acid in a test-tube. During its

excretion by the liver a part of the bilirubin is oxidised to the green pigment of the bile—**biliverdin**—but this differs merely in possessing two hydrogen atoms less in the molecule. Further coloured oxidation products are obtained by the action of strong nitric acid on these bile pigments—a fact that is the basis of the old Gmelin's test for bile.

It will thus be seen that **jaundice**, a condition in which there is an excess of bile pigment loose in the body, may arise not only by an obstruction of the bile duct but also as a result of over-activity of the cells of the reticulo-endothelial system, and further that in this way it is possible to have an abnormal increase of bile pigment without a corresponding increase of bile salts (with the metabolism of which the reticulo-endothelial cells are not concerned). This bile pigment becomes adsorbed on to the plasma proteins and so is not free to give its characteristic colour reactions—e.g. with diazotised sulphanilic acid (van den Bergh's reagent) until it has been liberated by precipitation of the proteins by alcohol. On the other hand bile pigment gaining access to the blood as a result of an obstruction of the bile passages of the liver will be necessarily accompanied by bile salts which will prevent the absorption of the bile pigment so that it will be free to give a purple colour directly with van der Bergh's reagent without any preliminary treatment with alcohol. Thus by chemical tests carried out on the blood serum it is possible to distinguish between jaundice of the obstructive and hæmolytic types.

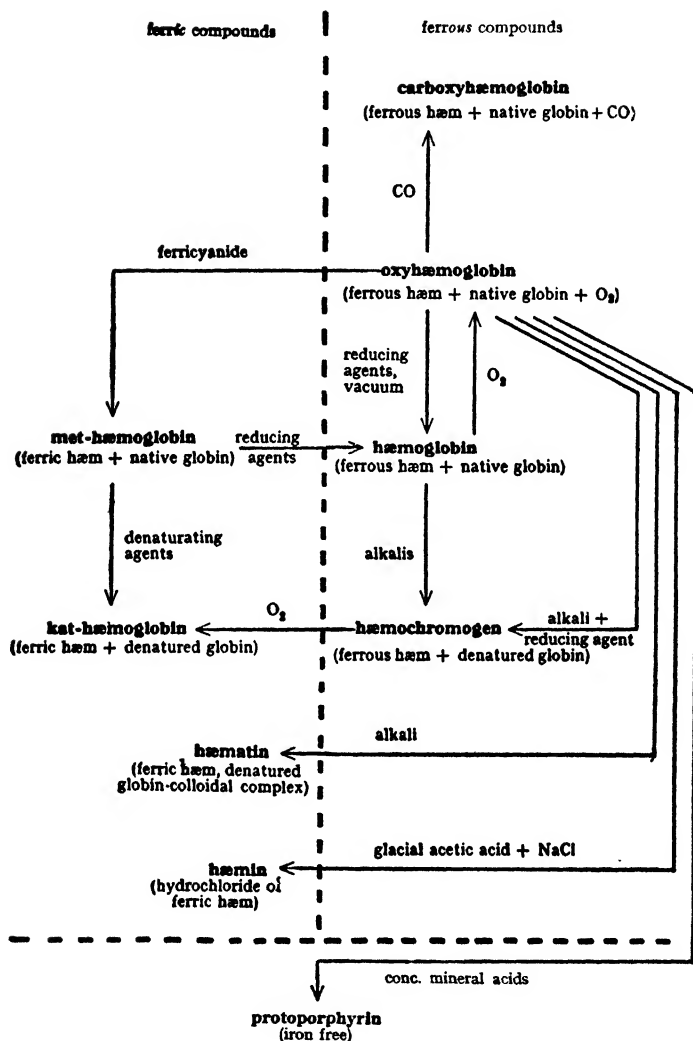
When the bile pigments pass into the alimentary canal they are attacked by the bacteria that abound in the intestinal contents. Apparently reduction then takes place, and nitrogen atoms are removed, the product

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being the brown pigment, **stercobilin**, of the fæces (Lat. *stercus* = dung). Of this pigment, however, a certain amount is absorbed into the blood stream, and is carried to the kidney, and so appears in the urine, where it is known as **urobilin**. This, it should be noted, is a pigment of only secondary importance in the urine, the most obvious yellow pigment being a substance called **urochrome**, which appears to be formed in the body from tryptophane but whose chemical constitution is unknown.

SUMMARY

It may assist the student if we give him in the following scheme the chief relationships between hæmoglobin and its derivatives. There is probably no need to remind him that he has the raw material for making them literally "at his finger tips," for with a knowledge of the chemistry of these substances specimens of them all can be obtained in a few minutes from a couple of drops of finger blood in half a test-tube of water.



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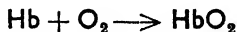
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CHAPTER XVIII

THE RESPIRATORY GASES

"Nor is there any chemical substance which exactly resembles hæmoglobin. . . . But for its existence man might never have attained any activity which the lobster does not possess, or had he done so, it would have been with a body as minute as the fly's."—*Barcroft*.

So far as the body is concerned, the most important property of hæmoglobin is its power of combining with oxygen in the lungs to form oxyhæmoglobin, and of liberating the oxygen again when this oxyhæmoglobin has been carried in the blood stream to active tissues where oxygen is needed. In this way the hæmoglobin subserves the essential function of transporting an abundant supply of oxygen to the internal organs which are cut off from direct contact with the external air. In this transport of oxygen between lungs and tissues we are dealing with a reversible chemical reaction. In the lungs it occurs in such a manner that oxyhæmoglobin is formed from its constituents, in a way which can be represented by an equation thus:—



if we allow the symbol Hb to stand for the whole complex molecule of hæmoglobin; while in the tissues the course of the reaction is reversed so that it is now to be represented as:—



Like all reversible chemical reactions, the direction in which this reaction will proceed is determined by the concentrations of the substances taking part. In the body the chief factor that determines whether oxyhæmoglobin shall be formed or shall be dissociated is the tension or concentration of oxygen. In the lungs the blood is exposed to a relatively high tension of oxygen and oxyhæmoglobin is formed—the oxygen is present in such concentration that it can force itself, so to speak, on the hæmoglobin. On the other hand, in the active tissues the oxygen is being used so rapidly that there is very little accumulated concentration of it, the oxygen molecules are present in such small concentration that they are powerless to resist the tendency of the hæmoglobin to throw them off, and therefore the oxyhæmoglobin now dissociates.

It is evident that in order to study the efficiency with which this transport of oxygen is carried out, that is, the amount of oxygen which each cubic centimetre of blood can carry between the lungs and the tissues—we must know not only the tensions of oxygen in lungs and tissues, respectively, but also the amount of oxygen taken up by the blood at each particular value of oxygen tension. We must expose blood to various tensions of oxygen, and when equilibrium has been obtained we must determine by means of analysis the amount of oxyhæmoglobin that has been formed in the blood at each particular tension of oxygen. On plotting these results we obtain a curve known as the dissociation curve of oxygen in blood, or more briefly the **oxygen dissociation curve of blood**, from which we can read off at a glance the amount of oxygen in a standard volume of blood at any particular value of oxygen tension and can

therefore easily determine the amount of oxygen which will be given off as the blood passes from a higher to a lower oxygen tension. Now ordinary atmospheric air contains about one-fifth of its volume of oxygen. Its tension, or partial pressure of oxygen will, therefore, be about $\frac{1}{5}$ of an atmosphere or $\frac{1}{5}$ of 760 millimetres of mercury, i.e. about 152 mm. Hg. Owing to the continuous absorption of oxygen by the blood, the air in the lungs will contain a smaller percentage, and, therefore, a smaller tension, of oxygen than the inspired air—not much above 100 mm. Hg. It is evident that since this is the highest oxygen tension that the blood meets in the body this will be the highest tension of the gas to which we need plot the oxygen dissociation curve if we are dealing with a normal body breathing normal atmospheric air. All the other values of oxygen tension that we shall need to use will be smaller than this, and will be obtained most conveniently by diluting ordinary atmospheric air to suitable extents with nitrogen in order to reduce the percentage and therefore the tension of oxygen in the mixtures. Suppose we have prepared a series of such mixtures possessing oxygen tensions ranging from small values up to about 100 mm. Hg. We then expose a small quantity of blood to each mixture by shaking in a suitable vessel—immersed in water at 37° C. if we wish to work at body temperature—and when equilibrium has been obtained we withdraw a measured sample of the blood and determine how much oxygen it contains in the form of oxyhæmoglobin by liberating and measuring the gas either by means of a vacuum pump or by treating with potassium ferricyanide (see p. 321). On plotting the results obtained in a series of such measurements—marking off values of oxygen tension along the horizontal direction and the total amount of

oxygen present in 100 c.c. of the blood vertically—we obtain curves such as are shown in Fig. 15:—

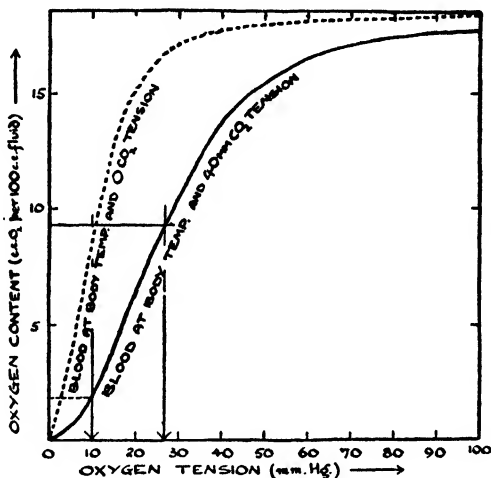
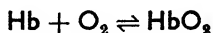


FIG. 15. Oxygen dissociation curves for blood (from Barcroft's data).

Leaving out of account for the moment the differences between these curves it will be seen that each of them has a complicated S-shape, the amount of oxygen taken up by 100 c.c. of blood increasing at first slowly and then more rapidly as the oxygen tension is increased, and, lastly, tending slowly to reach a maximum of about 18·5 c.c. of gas per 100 c.c. of blood at the highest values of oxygen tension investigated. Of course, of this oxygen a small amount is present in physical solution, as it would be in water, but this dissolved quantity amounts to only about 0·3 c.c. at 100 mm. Hg oxygen tension. So that, practically speaking, the whole of the oxygen carried by blood is in the form of oxyhæmoglobin.

This S-shaped curve is evidently the expression of a more complicated equilibrium than the simple reversible pairing off of the hæmoglobin and oxygen molecules that we have so far pictured. By applying the law of mass action to the equation



by assuming, that is, that the *product* of the concentrations of the reacting substances Hb and O_2 is in every condition of equilibrium of these substances proportional to the product of the concentrations of the products (in this case of HbO_2 only), we can calculate the concentration of oxyhæmoglobin that we should expect to be present in a solution of hæmoglobin of any given strength in equilibrium with any given tension of oxygen. The reader may perhaps care to try this for himself; he will then be easily able to convince himself that the oxygen dissociation curve to be expected on this assumption is not S-shaped but is a much simpler curve that rises ever more gradually from the origin to the maximum. It is, in point of fact, the curve known to the mathematicians as a rectangular hyperbola (Fig. 16). Now such a curve has undoubtedly been obtained in the past as the result of experiments on dialysed dilute solutions of hæmoglobin, and the S-shape of the oxygen dissociation curve of blood has been ascribed to some influence on the chemical properties of the hæmoglobin of the inorganic salts present along with it in the red corpuscles, but recent work in which special precautions have been taken to prevent "spoiling" of the hæmoglobin by bacterial decomposition or other agencies have yielded S-shaped curves for dilute solutions, and there is general agreement that such curves are given by the stronger solutions now rendered available by

improvements in the technique for crystallising hæmoglobin from blood.

It would seem from this that the property of giving an S-shaped dissociation curve is inherent in the hæmoglobin itself, and is not necessarily impressed on it by salts accompanying it in its solutions. But how then does the hæmoglobin come to give an S-shaped curve?

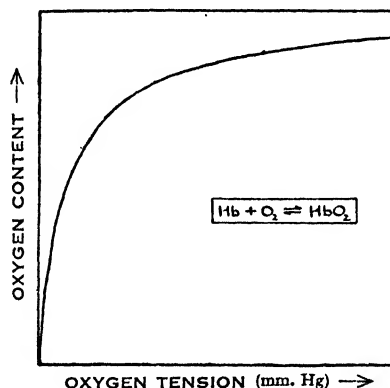
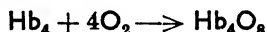


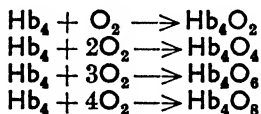
FIG. 16. Theoretical hyperbolic dissociation curve calculated for the equilibrium $\text{Hb} + \text{O}_2 \rightleftharpoons \text{HbO}_2$.

The first step towards the answer to this question is provided by the circumstance that recent accurate observations of the osmotic pressure of hæmoglobin solutions and measurements of the rate at which hæmoglobin settles out when its solutions are exposed to the enormous centrifugal forces developed in the high-speed "ultra"-centrifuge (p. 377) have independently shown that the molecule of hæmoglobin as it exists in solution is not Hb, but Hb_4 , i.e. the unit of hæmoglobin that combines with oxygen is the amount that contains not one atom, but four atoms

of iron, together, of course, with four porphyrin complexes and four molecules of globin. Seeing that it has been quite definitely known for a long time that hæmoglobin combines with one molecule of oxygen for each atom of iron it is evident that the complex Hb_4 will combine with 4 molecules of oxygen thus:—



But if we apply the law of mass action to this equation, owing to the fact that the oxygen concentration is involved four times, i.e. to the fourth power, the calculated oxygen dissociation curve for this equilibrium is much more S-shaped than any actually observed. It is therefore now supposed that what happens when hæmoglobin combines with oxygen is that it does so in four simultaneous but independent steps thus:—



The first of these equations represents a combination molecule for molecule and so its dissociation curve is hyperbolic; that of the second equation is slightly S-shaped; that of the third more so, and that of the fourth, as we have seen, excessively so. The mixture of all these equilibria simultaneously would then account for the intermediate degree of S-shapedness found in the experimentally determined oxygen dissociation curves of hæmoglobin solutions and blood.

While thus inorganic salts would appear to be of less importance than has previously been supposed in influencing the uptake of oxygen by hæmoglobin, there is no doubt of the significance of the effect on this process of the degree

of acidity or alkalinity of the solution in which the hæmoglobin finds itself. For example, by comparing the curves given in Fig. 15 it will at once be seen that in the presence of carbonic acid hæmoglobin combines with less oxygen at a given oxygen tension than in the absence of the acid. And what is here illustrated by carbonic acid is true of acids generally according to the several degrees of acidity they produce. Now the point of equilibrium attained by hæmoglobin and oxygen is determined by the relative rates of the forward combination of these two substances and of the backward dissociation of oxy-hæmoglobin into its components, and as the acid changes this point of equilibrium it must affect this ratio—it must, that is, affect differently the rates of the forward and backward reactions. We described a precisely similar state of affairs in connection with the hydrolysis of esters in Chapter XIV, but whereas the equilibrium in the ester system might require days or even weeks for its attainment the speeds of the forward and backward reactions in the hæmoglobin system are immeasurably greater and the equilibrium is attained in a few hundredths of a second. Or rather not quite “immeasurably,” for it has proved possible to measure them by squirting solutions of hæmoglobin and oxygen (or, for the reverse reaction, solutions of oxyhæmoglobin and sodium hydro-sulphite) at such a rate through an efficient mixing chamber that the mixture can be passed into a long observation tube before any appreciable amount of chemical action has taken place. The progress of the reaction can be followed by spectroscopic observation of the colour at a series of points along the tube corresponding to time intervals calculable from the known rate of flow of the mixture.

By this ingenious method it has been shown that the effect of acids on the oxygen-hæmoglobin equilibrium is due entirely to their influence in accelerating the decomposition of oxyhæmoglobin; they do not affect the rate of uptake of oxygen by the hæmoglobin at all. And the same is true of increase of temperature; it tends to loosen the firmness with which hæmoglobin holds its oxygen entirely by accelerating the rate of decomposition of oxyhæmoglobin.

Now this influence of acidity and of temperature in tending to dissociate oxyhæmoglobin is of considerable importance to the body. The whole point, if we may so express it, of the combination of oxygen with hæmoglobin is its reversibility. Hæmoglobin not only combines with oxygen in the lungs, but it parts with it in the tissues. Many substances of course combine readily enough with oxygen—metallic iron itself does—but it takes a blast furnace to remove the oxygen again. When, however, the iron is combined in the hæmoglobin molecule it holds the oxygen only with sufficient firmness to enable it to transport it from the lungs and unload it in the tissues where it is required. And in this it is assisted by the elevated temperature of the body, and by the carbonic and other acids that enter the blood, to an extent that can be judged by following the curves of Fig. 15. At the relatively high tension of oxygen to which the blood is exposed in the lungs it is almost as nearly completely saturated with oxygen whether it contains carbonic acid or not. In other words, the acid does not appreciably reduce the amount of oxygen that the blood takes up in the lungs. Now when the blood passes through the capillaries in active tissues and there gives up its oxygen the tension of oxygen naturally falls; by the time the

blood has given up about half its oxygen, as indicated by the horizontal line, the tension in the blood devoid of carbonic acid will have fallen to 10 mm. Hg, while the oxygen tension in the blood containing the usual amount of carbonic acid will still be 26 mm. Hg. In other words, the carbonic acid normally present in the blood tends to maintain the *tension* of the oxygen remaining in the blood, and as it is this tension that is the driving force for the diffusion of oxygen through the capillary walls into the tissues it will be realised that the properties of hæmoglobin are modified by the acid in such a direction as to assist in that efficient transport of oxygen on which the vigorous life of a warm-blooded creature depends.

Before we leave the question of the carriage of oxygen by the blood we should mention that, in plotting the oxygen dissociation curves we have given, we have expressed the total quantity of oxygen present in the blood under any given conditions in c.c. of gas per 100 c.c. of blood. This method has the merit of clearness, but it is usual to find oxygen dissociation curves in which the quantity of oxygen present is expressed as a percentage of the total amount of oxygen with which the blood will combine at a high oxygen tension. This is called the **percentage saturation** of the blood with oxygen. This method of expressing the total oxygen content of blood is adopted because it is somewhat easier to measure the relative degree of saturation of the hæmoglobin with oxygen than the total actual volume of oxygen present. But this nomenclature should cause no difficulty when it is remembered that in normal blood a percentage saturation of 100 corresponds to a total actual content of oxygen of 18.5 c.c. per 100 c.c. of blood, and soon in proportion.

The study of the carriage of carbon dioxide by blood follows the same lines as the investigation of the carriage of oxygen. We can plot out **carbon dioxide dissociation curves** for blood by methods analogous to those used to obtain the oxygen dissociation curves. We expose blood to increasing tensions of carbon dioxide and then estimate the total amount of carbon dioxide present per 100 c.c. of blood by withdrawing a measured sample and extracting the carbon dioxide by means of the vacuum pump or by expelling it from the blood by adding acid. Seeing that carbon dioxide exerts so powerful an influence on the oxygen dissociation curve of blood it is not surprising that oxygen should produce a marked effect on the course of the carbon dioxide dissociation curve. Indeed, it is found that the actual carbon dioxide dissociation curve given by a sample of blood depends on whether or not the blood contains oxygen. If it be free from oxygen it will take up appreciably more of carbon dioxide at a given tension of the gas than if it be completely oxygenated and exposed to the same carbon dioxide tension. We thus obtain two carbon dioxide dissociation curves, one for reduced and one for fully oxygenated blood, of which the latter runs at a certain distance below the curve for the reduced blood throughout its course. These two curves are shown in Fig. 17. In this effect of oxygen on the carrying power of blood for carbon dioxide we see an adaptation which enables the carbonic acid to be carried from the tissues and to be discharged from the lungs at a greater rate than would otherwise be possible. For as the blood becomes reduced in the active tissues it becomes the more capable of combining with carbon dioxide. On the other hand, the oxygen taken up by the blood in the lungs tends to expel the carbon dioxide—oxygenated

blood being capable of combining with less carbon dioxide than reduced blood at the same carbon dioxide tension. We have already mentioned that the carbon dioxide tension in the arterial blood is about 40 mm. Hg, and this is the lowest tension of carbon dioxide which the blood ever possesses in the normal body. From the dissociation curves just given it is seen that at this carbon dioxide

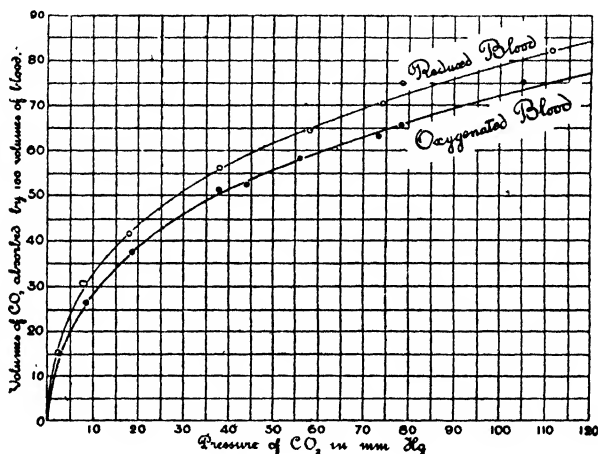


FIG. 17. The carbon dioxide dissociation curves for oxygenated and reduced blood.

Note the extra carbon dioxide taken up by the reduced blood at all tensions of the gas. (From Christiansen, Douglas and Haldane.)

tension 100 c.c. of the fully oxygenated blood contain over 50 c.c. of carbon dioxide, so that even arterial blood, which contains less carbon dioxide than any other blood in the body will give off half its volume of this gas on acidification. Of this gas a certain small fraction will be in physical solution, but even though carbon dioxide is a fairly soluble gas, at the tensions at which we are working

this dissolved portion will not amount to a very appreciable fraction of the whole of the gas taken up. It is evident, therefore, that the gas combines chemically with some constituent of the blood just as does oxygen. There has been considerable discussion as to the exact mode of combination of carbon dioxide in blood, but there is good evidence for believing that at least a considerable fraction of the combined portion is in the form of sodium bicarbonate. We shall discuss this evidence more fully on a later page (p. 411); at present we shall state simply that the sodium required for the formation of this sodium bicarbonate is supposed to come mainly from the molecule of hæmoglobin, which is a weak acid, and exists as its sodium salt in the weakly alkaline blood. When this is acted upon by carbonic acid, the sodium is removed from the hæmoglobin and goes to form sodium bicarbonate; in the lungs the carbonic acid escapes, and the sodium salt of hæmoglobin is once more formed. The reason why oxygenated blood takes up somewhat less carbon dioxide than does reduced blood at the same carbon dioxide tension is that oxyhæmoglobin is a somewhat more powerful acid than reduced hæmoglobin, and so holds more firmly to the sodium, and gives off less of it to form bicarbonate with the carbonic acid. In both cases, however, some of the bicarbonate ions formed in the red corpuscles by the onisation of the sodium bicarbonate diffuse out into the plasma so that the combined carbon dioxide of the blood is not confined to the corpuscles as is the combined oxygen.

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CHAPTER XIX

SOME APPLICATIONS OF PHYSICAL CHEMISTRY: GAS TENSIONS: OSMOTIC PRESSURE

"I am convinced that biological chemistry cannot develop into a real science without the aid of the exact methods offered by physical chemistry."—*Arrhenius*.

Gas Tensions

IN our discussion of the carriage of oxygen and carbon dioxide by blood we frequently referred to the tension exerted by a particular constituent of a gas mixture, or to the tension of a gas in a liquid, but we did not turn aside from our main argument in order to explain and define these terms. The tension or partial pressure of a gas is that property which determines whether it shall diffuse into or escape from a space to which it has access. If the tension of a gas at any place is high, then the gas will tend to pass by diffusion to places where the tension is lower; conversely, a region of low tension will receive the gas from a situation where a higher tension prevails. The tension of a gas is therefore a factor analogous to temperature; for it is the relative temperatures of bodies which determine the direction of flow of heat between them.

Now, according to modern kinetic theory, this tendency to diffusion shown by a gas is an expression of the ceaseless energy of movement of its molecules, and the tension it exerts is the sum total of all the small elements of pressure exerted by the separate molecules as, in their movement,

they happen to collide with the walls of the space in which the gas is contained. If we have a mixture of gases in a given space, then the tension of each constituent is determined by the total rate of bombardment of the confining walls by molecules of that particular constituent. Now it is evident that the rate of bombardment by a molecule of any particular kind depends upon the number of such molecules present in each cubic centimetre of the gas mixture, and that this number may be varied in two ways—either by varying the composition of the gas mixture while keeping its total pressure constant or by changing the total pressure, and so, for example, compressing into a smaller space a greater number of molecules of the particular constituent in which we are interested, and, incidentally, of the other constituents as well. It therefore follows that the tension exerted by any one of a mixture of gases is determined both by the relative amount of that particular gas present in the mixture, and also by the total pressure exerted by the mixture. In symbols we may say that if the percentage by volume of the given constituent of the mixture be x , and the total pressure exerted by the mixture be p , then the tension of the particular constituent will be x per cent. of p . And this tension will be exerted unchanged, no matter how many molecules of other gases may be present in the mixture as well. For the spaces between the molecules of gases are so large that the molecules of one gas may move between the molecules of a second constituent without mutual interference—at least at moderate pressures.

In the case where one constituent of a gas mixture is the vapour of a liquid the same considerations hold; the tension exerted by the vapour is determined by the

relative proportion of it present. But in the case where there is also some of the liquid itself present, when, as it is said, the vapour is saturated, then the tension exerted by the vapour is no longer variable; it is a fixed quantity, which is determined solely by the temperature. If we attempt to increase the tension of vapour, say by compression of the mixture, the excess of vapour condenses, and the amount of liquid is increased; on the other hand, if the tension of the vapour is below the saturation value, then some of the liquid already present evaporates until the saturation value is reached. The tension exerted by the vapour therefore remains constant as long as there is some of the liquid present. These considerations are important in dealing with gases that are saturated with water vapour, such as air expired from the lungs, or air that has been exposed to blood and other aqueous fluids during experiments. The amount of the saturated water vapour pressure corresponding to the prevailing temperature must be subtracted from the total pressure exerted by the moist mixture, and only the remaining pressure is to be shared between the other constituents, according to their relative percentages by volume, in order to determine their respective tensions.

To take a numerical example—suppose that of the total volume occupied by the oxygen, nitrogen and carbon dioxide in a sample of alveolar air, 6 per cent. is occupied by the carbon dioxide. Suppose also that the barometer stands at 760 mm. Hg. In the lungs this air was saturated with aqueous vapour at body temperature (37° C.), since it was in contact with the moist lung surface. It is known that at this temperature the tension exerted by saturated aqueous vapour is 47 mm. Hg. Therefore the remaining tension to be distributed between

the oxygen, nitrogen and carbon dioxide is $760 - 47 = 713$ mm. Hg. As the carbon dioxide forms 6 per cent. of this mixture of the three gases, the tension it exerts will be, therefore, 6 per cent. of 713 mm. Hg, which is about 42.8 mm. Hg.

In defining the tension of a gas dissolved in a liquid the same considerations apply as in the case where it forms a constituent of a gas mixture. The tension of the gas is made up of the pressure exerted by its molecules as they strike the liquid surface. In order to arrive at a quantitative expression let us consider what happens when a liquid containing a gas in solution is brought into contact with a space also containing the gas. If the tensions of the gas in the liquid and in the gas space are such that in a given time more gas molecules strike and pass through its surface from below than from the gas space above, there will be a tendency for more gas molecules to pass through the surface from liquid to gas than pass into the liquid from the gas. The liquid therefore loses some of the gas to the gas space. On the other hand, if the tensions in the two phases are such that more gas molecules strike and penetrate the surface from above than from the liquid below, then more molecules will pass from the gas space into the liquid than from liquid to gas; in this case the liquid gains gas from the gas space. But for a certain concentration of gas in the liquid, the number of molecules passing in a given time through the surface in one direction is just equal to the number passing in the same time in the opposite direction, so that no apparent change in the distribution of the gas between the two phases takes place. When this condition of equilibrium is fulfilled the tension of the gas in the liquid is said to be equal to that in the gas

space. The tension of a gas in a liquid is therefore defined as being equal to the tension of the gas in the gas space with which the liquid is in equilibrium. Measurements of the tensions of gases in biological fluids are important in physiological studies; for example, an accurate knowledge of the tensions of the gases in arterial blood is of prime importance in deciding the nature of the activity of the lung membrane. For if it should happen that under any conditions the tension of oxygen in the arterial blood leaving the lungs becomes greater than that in the alveolar air on the other side of the lung membrane, it is certain that any oxygen which then passes into the blood from the alveolar air cannot pass by a process of physical diffusion; it must be forced up the slope of oxygen tension by some active process for which energy is supplied by the cells forming the lung membrane itself. We do not wish to discuss this topic more fully here; we will content ourselves with a reference to the principle of the methods by which these measurements are made. It follows at once from our previous considerations. The liquid is shaken up or otherwise efficiently exposed to a small bubble usually of air until, by diffusion, the gases in the bubble have come into equilibrium with those in the liquid. Then all that is necessary is to analyse the bubble and to calculate from the results of the analysis the tensions of the various gases in it; the tensions of these gases in the blood or other liquid are equal to those in the equilibrated bubble.

Osmotic Pressure

We have many reasons nowadays for believing that there exists some kind of attractive force between the molecules of a dissolved substance and those of the

solvent in which it is dissolved—an attractive force which has to be overcome whenever we separate some of the pure solvent from the solution. For this involves the separation of solvent molecules from the molecules of dissolved substance which are left behind in the more concentrated residue of the solution. Suppose, for example, that we have some salt dissolved in water, and we consider the separation of the water by spontaneous evaporation. We find that the vapour pressure of the solution at any temperature is appreciably less than that of pure water at the same temperature, the difference being a measure of the energy with which the molecules of salt attract the molecules of water. Again, suppose we wish to separate the solvent by boiling, then we find that in consequence of the diminished vapour pressure of the solution we have to heat it to a higher temperature than the boiling point of pure water in order that its vapour pressure shall be equal to that of the atmosphere, and that the liquid shall, in consequence, boil. This **elevation of the boiling point** is, then, another measure of the amount of work required to separate the water from its dissolved solid. Lastly, if we cool the solution, pure ice at first separates out. This constitutes yet another method of separating pure solvent from the solution, and again we find that there is greater difficulty in obtaining the ice from the solution than from the pure solvent, for the solution requires to be cooled to a temperature appreciably below 0°C . before it begins to freeze. We have therefore a **depression of the freezing point** of the solution below that of the pure solvent, a depression which is yet another measure of the work required to be done in separating the salt from the water molecules.

Lastly, we can, theoretically, at least, separate the water from the salt solution by means of pressure, provided we can find some membrane that will act as a kind of sieve and will allow molecules of water, but not molecules of salt, to pass through it. Then we can apply pressure to the solution on one side of the membrane, and by this means cause water to pass through, leaving a stronger solution behind. One can imagine some such arrangement as is shown in Fig. 18, in which the special **semi-permeable membrane** forms the bottom of a cylinder in which the solution can be submitted to pressure by means of a piston. In this process

of pressure filtration, just as in the process of evaporation already mentioned, we have to perform work in separating the pure solvent from the solution; in this case this is shown by the fact that we have to exert extra pressure on the piston over and above that which would be required to force water through the membrane if the cylinder were

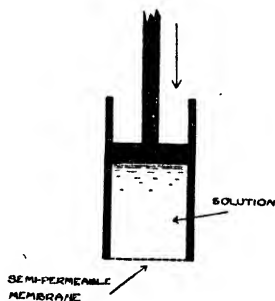


FIG. 18.

filled at the beginning with pure water instead of a salt solution. The extra pressure that has to be exerted is termed the osmotic pressure of the salt in the solution. This osmotic pressure is an appropriate subject of study for the physiologist, because we find that most processes of secretion involve the separation from the blood, through a membrane or a cell, of a solution either more or less dilute with regard to at least some constituents than the blood which is supplied on the other side of the membrane.

The existence of this osmotic pressure makes itself evident in another way. Suppose we have our solution in a cylinder the end of which is closed by a semi-permeable membrane, as before. Let us, however,

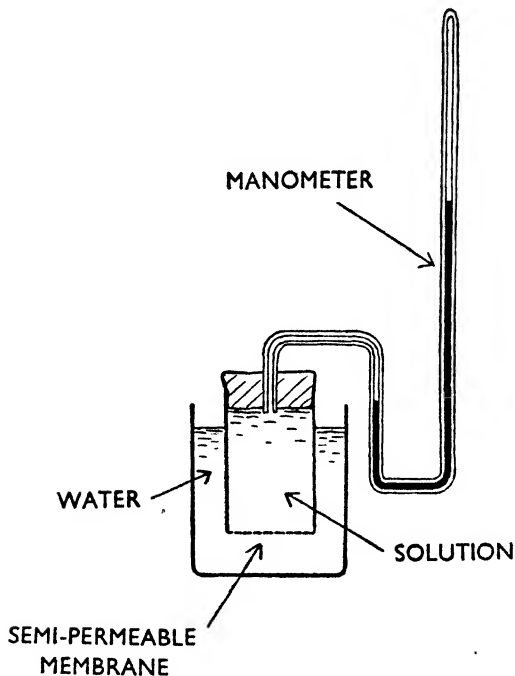


FIG. 19. Apparatus for direct measurement of osmotic pressure.

discard the piston, and instead close the upper end of the cylinder with a cover which carries a manometer of some kind. The cylinder being filled with a salt solution, let the whole then be immersed in a vessel of pure water as in Fig. 19. Under these circumstances diffusion of water

molecules will take place through the membrane, which is supposed to be freely permeable to them. Some molecules will diffuse into the cylinder from outside; others will tend to pass out into the surrounding mass of fluid. If we had pure water both inside and outside the cylinder we should have as many water molecules diffusing in as diffusing out, and the whole system would remain in equilibrium, no pressure change being recorded by the manometer. But in the case where the cylinder contains a salt solution, the water molecules inside will be at a disadvantage, for they are attracted back into the cylinder by the salt molecules which themselves cannot get through the membrane. Thus it comes about that more water molecules will diffuse into the cylinder in a given time than will diffuse out. There will, therefore, be an accumulation of water inside the cylinder which will give rise to an increase of pressure. But this increase of pressure will tend to squeeze out mechanically more water molecules through the membrane. So that the increase of pressure in the cylinder will continue until the assistance given by the pressure to the inside water molecules is just sufficient to compensate for what they lose owing to the back attraction of the salt molecules. Equilibrium will then be obtained, the inside water molecules, assisted by the pressure, escaping from the cylinder in equal numbers to those which enter. The constant pressure now registered by the manometer is thus equal to the pressure required to separate the inside water molecules from their accompanying salt molecules; it is, then, the osmotic pressure of the salt in solution. This is the principle of the method by which osmotic pressure is directly measured. In actual practice the cylinder we have described is furnished by an ordinary unglazed

porous pot; it is found that this can be rendered semi-permeable—at least, in the case of some solutions—by precipitating in its pores a layer of copper ferrocyanide, by filling it with copper sulphate solution and immersing it in a solution of potassium ferrocyanide. The two solutions meet by diffusion in the middle of the wall of the pot, and there react with the formation of a layer of the copper ferrocyanide precipitate which forms a semi-permeable membrane supported by the rigid earthenware. After thorough washing it is necessary merely to fit a stopper and a suitable manometer and the apparatus is ready to give direct readings of the osmotic pressure exerted by any dissolved substance that will not pass through the membrane.

Many living cells seem to be bounded by membranes which, if not completely semi-permeable, are very limited in their permeability to many dissolved solids. Red blood corpuscles furnish us with an example. When these are placed in a salt solution that is more dilute than their contents, water passes into them—osmotically—and they swell and ultimately burst. The hæmoglobin is thereby liberated into the surrounding liquid, with the result that the mixture possesses a transparent red colour, and comes to differ very markedly in appearance from the original opaque blood. When the red corpuscles have been broken up in this fashion the blood is said to have been laked. It should be mentioned that this osmotic process is not the only method whereby blood corpuscles can be laked. The corpuscle membranes can be ruptured not only by bursting owing to treatment with a hypotonic solution, but also by many reagents which will attack them chemically. Ether and saponin may be mentioned among these. The venom of some snakes

produces extensive hæmolysis in a similar fashion. Then again, the sharp points and edges of the ice crystals formed when blood is frozen pierce the outer membranes of the red corpuscles and liberate their contents, so that alternate freezing and thawing finally results in the complete laking of the blood.

On the other hand, if the blood corpuscles are placed in a strong salt solution, water is attracted out through their membranes, with the result that they shrink. There is, however, one particular molecular concentration of salt which will not cause a change in the water content of the corpuscles, so that their volume remains unchanged. Such a solution is said to be **isotonic** or **isosmotic** with the corpuscles. If made up with sodium chloride it is usually referred to as "normal saline"; it will contain about 0·6 per cent. of the salt if it is isotonic with frogs' corpuscles, and a larger concentration—0·95 per cent.—if it is to be suitable for those of a mammal. In this they will remain in a normal condition. And what we have said with regard to blood corpuscles is true of cells in general. From this it will be evident that one of the chief requirements of a fluid which shall maintain in as nearly normal a condition as possible the structure and functional activity of a tissue will be that it shall possess a suitable osmotic pressure.

A little further consideration of this behaviour of red blood corpuscles to sodium chloride solutions will make it evident that the salt cannot penetrate the limiting membranes of these cells. For if it could, equality of concentration would ultimately be established inside and out and there would then be no effective osmotic pressure to balance that of the other cell contents, and so the corpuscles would become laked in a solution of sodium

chloride as they do in water. But as the corpuscles remain intact in a sodium chloride solution of the appropriate strength we conclude that this salt cannot enter the cells. On the other hand, when we repeat the experiment with a solution of ammonium chloride we find that the corpuscles lye in all concentrations of this substance, and we conclude from this that the cell membranes must be readily permeable to both ammonium and chlorine ions. The reason why the sodium chloride does not pass into the corpuscle must be, therefore, that the membrane is not permeable to the sodium ions. And if the sodium ions cannot penetrate the membrane their partners the chlorine ions also will be prevented from entering, for any separation of the oppositely charged ions would set up powerful electrostatic forces against which further ionic movement would be impossible. Here, then, we have a particular case of the important general principle that it is not necessary that a membrane should be impermeable to both ions of a salt in order that it should prevent the passage of the salt as a whole; if the membrane is impermeable to only one of the ions, the other will also be prevented from entering. We shall discuss this further in the next chapter.

The membranes we find in the body differ in several respects from inorganic precipitation membranes, such as the one we have just described. In the first place they are not often so completely impermeable to dissolved crystalloids as is copper ferrocyanide, for many secretions contain salts in somewhat the same concentrations as in blood. And again, an animal membrane is usually a cellular structure whose semi-permeability depends not on mere physical properties, but on active processes occurring within the cells of which it is composed.

That is to say that the cells may utilise energy obtained from their own oxidative processes for facilitating or retarding, as the case may be, the passage of particular substances through their walls.

Of course, in any case where a particular constituent of a solution is not kept back by a membrane, but comes through in unaltered concentration, it is evident that since no concentration of this substance has taken place there has been no separation of solvent from this particular solute, and therefore no energy has had to be supplied in order to overcome that part of the osmotic pressure due to this constituent. We mention this because this is one of the conditions which we believe to obtain in the glomeruli of the kidney. The membrane separating the blood in the glomerular vessels from the cavity of Bowman's capsule is supposed to be impermeable to the blood proteins, but to be capable of allowing the unrestricted passage of salts, sugar and urea. If the glomerulus, then, is to act merely as a passive filter, whose driving force is the blood pressure, it follows that it is the osmotic pressure of the blood proteins only which is the opposing force to the filtration. More exactly we should say that it is the osmotic pressure of the plasma proteins, since the hæmoglobin is confined to the corpuscles, and so is not free to be filtered off in any case.

Now the osmotic pressure exerted by the plasma proteins has been measured directly. For this purpose it was not necessary to use a very special membrane, as most animal membranes are impermeable to proteins, although, in the dead condition at all events, they are freely permeable to salts. Parchment serves excellently for the purpose. If an osmometer be constructed of this material and filled with blood plasma it is found that at first on immersing it

in water a fairly high osmotic pressure is recorded. But the value of this pressure gradually falls as the salts originally present in the plasma diffuse through the membrane to the outside. After a time, however, when this diffusion has come to a standstill, the osmotic pressure will remain steady, its value being that due to the indiffusible proteins of the plasma, the salts and other diffusible substances being now in equal concentrations on both sides of the membrane, and so producing no effect. The value of the osmotic pressure so obtained is very small compared with that exerted by salt solutions of ordinary strengths. It amounts to about 30 mm. of mercury—three centimetres only.

This, then, is an estimate of the pressure that will oppose filtration from the blood plasma of a solution containing its crystalloid substances in unaltered concentrations, but from which the plasma proteins have been separated by the filtration membrane. On the other hand, the pressure available for the filtration is the difference of pressure of the blood in the glomerular capillaries and that of the urine in the cavity of the capsule. The pressure in the capillaries will be but little below the general blood pressure, particularly as these are relatively wide vessels which narrow considerably only as they leave the glomerulus. The pressure of fluid in the capsule will be but small, for the contractions of the ureter remove the urine as fast as it is formed. We conclude, therefore, that under normal circumstances the available filtration pressure will be able easily to overcome the opposing osmotic pressure of the plasma proteins so that the glomerulus and capsule together constitute a pressure filtration apparatus. It has been found that when the effective filtration pressure is reduced

—as it may be by a fall of blood pressure or by an increase of pressure on the ureter side of the glomerular membrane—then the formation of urine comes to a standstill when the ureter pressure has risen to within 40 mm. Hg of the blood pressure; that is to say, when the effective filtration pressure is just about balanced by the osmotic pressure of the plasma proteins. From this it is concluded that the main process in the formation of urine is one of filtration, for which the necessary energy is supplied by the heart in maintaining the blood pressure. It is also evident that with a given blood pressure the filtration will be the easier the lower the osmotic pressure of the plasma proteins. Now the osmotic pressure exerted by any substance in solution is, in general, very nearly, and in the case of dilute solutions, practically exactly, proportional to its concentration. If then we inject a considerable quantity of saline solution into the blood, the blood pressure may be kept constant by the regulatory activity of the vasomotor centre, but nevertheless a much increased formation of urine takes place owing to the fact that the saline has diluted the plasma, and so has reduced the osmotic pressure which its proteins oppose to the filtration. It is interesting to find also that this saline diuresis is not accompanied by any increase in the amount of oxygen used per minute by the kidney, which shows that the energy used in the extra filtration is not derived from oxidations in active kidney cells, but is obtained from a source external to the kidney, i.e. the heart-beat. The student will now easily realise why, in order to compensate for the great fall of blood pressure occurring during conditions of shock, it is useless to inject a plain saline solution. This would dilute the plasma and so reduce the osmotic pressure which its proteins oppose

to the filtration in the glomeruli. The filtration would therefore become easier, and increased formation of urine would continue until all the excess of fluid had been removed again. But suppose that to the saline we add some colloid that will not pass through the glomerular membrane and that will exert an osmotic pressure about equal to that exerted by the plasma proteins, then on injection of this mixture there will be no diminution of the force opposing the filtration, and therefore no increased formation of urine. The injected fluid will therefore remain in the blood vessels and so will assist in maintaining the blood pressure and the necessary circulation of the blood corpuscles for a considerable time. A suitable non-toxic colloid for the purpose is furnished by gum arabic; so that **gum-saline** injections have found extensive applications in combating the circulatory disturbances caused by wounds. We mentioned that the osmotic pressure of the proteins of the plasma amounted to about 30 mm. Hg. The osmotic pressure of the gum in the injection fluid should also have this value if the normal conditions are to be reproduced as accurately as possible.

It is evident that these considerations are important in connection with the passage of fluid not only through the kidney glomeruli as a first step in the formation of urine but also through the capillaries of the body generally as a first step in the formation of lymph. Here also the driving force for filtration will be the mechanical pressure of the blood on each point of the capillary wall; this will be the greater, the greater the degree of dilatation of the capillary and, consequently, the greater the share of the general blood pressure it bears. Here also the back osmotic attraction exerted by the proteins of the plasma on the

exuded fluid must be overcome before any filtration occurs at all, and even then its rate is determined by yet another factor, namely, the permeability of the capillary wall itself. For a detailed description of the circumstances under which these three factors vary in the body, the student should consult his textbooks of physiology.

When we turn from this study of the mode of formation of urine and lymph to consider the behaviour of such secreting organs as the salivary glands we at once meet with activities that cannot be explained in terms of a simple process of filtration. For example, when we find that an active submaxillary gland can secrete saliva until the pressure registered on a manometer inserted into its duct is far above the pressure of blood in the arteries supplying the gland, it becomes obvious that the secreting cells must be drawing on the energy produced during their metabolism in order to be able to perform the work of transferring water from the blood to the secretion at a higher pressure in the duct.

In concluding this account of osmotic phenomena we wish to refer once again to the magnitude of the osmotic pressure of the proteins of blood plasma, in order to point out that these small values of about 30 mm. Hg are very much smaller than those observed in the case of solutions of crystalloid substances such as sugars and salts. This is because the osmotic pressure exerted by any substance in solution is practically proportional to the number of molecules of it present per unit volume of the solution, but is independent of their size or kind. In the case of proteins and gum where the molecules are large there will be but relatively few of them in unit volume of solutions of ordinary strengths, therefore the osmotic

pressure exerted by these will be low. On the other hand since salt molecules and sugar molecules are comparatively small, even a small weight of any of these substances will be made up of a large number of molecules, so that their solutions will contain a relatively great concentration of molecules, and so will exhibit a high osmotic pressure. As a matter of fact, a substance in solution behaves just as if it were a gas occupying the same volume as the volume of the solution. The osmotic pressure exerted by it in the solution is identical with that which it would exert if it were converted into a gas occupying a volume equal to that of the solution and at the same temperature. Now a gram-molecule of any gaseous substance which is made to occupy a volume of 22.4 litres exerts a pressure of just one atmosphere at 0°C . Similarly a gram-molecule of any substance dissolved in a volume of 22.4 litres of solution exerts an osmotic pressure of one atmosphere at 0°C . In other words a solution containing one gram-molecule in a litre will exert an osmotic pressure of 22.4 atmospheres at 0°C . since the osmotic pressure is proportional to the concentration. In the case of an electrolyte, however, the value will be even higher than this because the splitting up of some of the molecules into their ions leads to an increase in the total number of particles in solution, and an ion is just as capable of producing its share of the osmotic pressure as is a whole molecule.

We have mentioned that both the vapour pressure and the freezing point of a solution are depressed below those of the pure solvent by an amount dependent on the total molecular and ionic concentration of the solution. The determination of the freezing point has therefore been much used as a means of estimating the total osmotic

concentration of biological fluids, but the accuracy attained is not high and in any case such a determination tells us nothing as to the relative concentrations of the various molecules and ions present, for in dilute solutions at all events the osmotic effect depends only on their total concentration and not on their kind. Recently extremely delicate thermo-electric methods have been applied to the accurate determination of vapour pressures, and have been used for such purposes as the accurate estimation of the change in total osmotic concentration occurring when blood takes up CO_2 (which is found to be less than would be expected if all the CO_2 taken up were converted to bicarbonate) or a muscle contracts (which is found to be greater than can be accounted for by the sum total of chemical changes at present known to occur in a muscle during activity). The same method has been applied for observing the depression of vapour pressure that occurs when a known amount of a solute is dissolved in a complicated body fluid or tissue, and so for obtaining information as to the relative proportions of the total water of the tissue that are respectively "free" to act as a solvent or in some way "bound" so as not to be available in this respect.

It is often found difficult to realise the relation between the process of diffusion of a dissolved substance throughout a mass of solvent and the osmotic pressure it exerts when confined by a semi-permeable membrane. Possibly the matter may be made clearer by the following consideration. The fact that a dissolved solid can diffuse throughout a mass of liquid in which it is soluble is due to the same attraction between solute and solvent which we found to be shown by the existence of osmotic pressure. In the absence of any membrane, not only the solvent molecules

but also the molecules of dissolved solid are free to move in response to this attraction. They therefore diffuse out among the solvent molecules, until equality of concentration is attained throughout the bulk of the solution. The system then remains in equilibrium, its free energy having become a minimum. On the other hand, when the dissolved solid is held inside a membrane, through which it cannot diffuse, the attraction between it and the molecules of its solvent can lead to the movement of solvent molecules only. These are drawn inside the membrane, with the result that an increased pressure—the osmotic pressure—is set up. It is important to realise that the tendency to equalisation of concentration of a dissolved substance will be manifested in the body fluids of organisms, and that any process in which this tendency is resisted, by which differences of concentration are produced in a solution, necessarily requires the expenditure of energy in the part of the tissue which brings about the change. Of course, in the organism, processes of diffusion are often accelerated by osmotic effects. For, if we have a case where a solution of a crystalloid is separated, by a membrane through which it can readily pass, from a solution of a colloid which will not pass through the membrane, then as the water is drawn by osmotic attraction into the colloidal solution, the dissolved crystalloids will be so drawn in as well. But if the crystalloids are at all hindered in their diffusion by the membrane, then the solution will not be absorbed so rapidly, for the osmotic pressure of the dissolved material now opposes the process of absorption. These considerations are important in connection with the discussion of the mechanism of absorption from the alimentary canal, where the digested food represents the solution of crystalloids separated by the intestinal

epithelium from the solution of colloids—the blood plasma—in the capillaries of the villi. But in this case also, as in that of secretion, we meet the complication that the cells concerned are capable of producing energy which they can utilise for overcoming the laws governing diffusion and osmosis in non-living systems. An absorbing cell is merely a secreting cell turned back to front. This could be no more strikingly illustrated than by the fact that a piece of living intestinal mucous membrane set up as an osmometer membrane will even transfer and pile up isotonic saline solution from one side of itself to the other—an achievement to which only a living energy-yielding membrane could attain.

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CHAPTER XX

FURTHER APPLICATIONS OF PHYSICAL CHEMISTRY:
COLLOIDS: ADSORPTION: THE REACTION OF BODY FLUIDS:
THE FUNCTIONAL IMPORTANCE OF ELECTROLYTES

Colloids: Adsorption

"The colloid possesses ENERGIA. It may be looked upon as the probable primary source of the force appearing in the phenomena of vitality."—*Graham*.

It is usual to regard a solution of a simple crystalline substance such as urea or glucose as representing the simplest type of solution with which we are acquainted. One imagines the molecules of the dissolved substance to be distributed singly between those of the water or other solvent, and to be in constant movement like the molecules of a gas. In the case of dissolved salts the system is more complex because the salt will in general be split up to a considerable extent into its component ions. But also in this case the dissolved molecules and ions are small and active. But when one comes to consider a protein or a starch it is easy to see that a solution of such a substance differs very much from one of urea or of a salt, inasmuch as the ultimate particles of a dissolved protein are no longer small and active, but large and therefore sluggish. For this reason they diffuse through water at a rate which is very small compared with that at which the crystalline substances will travel. This observation was first made by Graham, who classified all soluble substances into two classes—those which were characterised by the property of rapid diffusion and those which lacked this property. Most of the members of the first group are crystalline

substances; Graham therefore called these the **crystalloids**. On the other hand he invented the name **colloids** for the slowly-moving substances, after the Greek for a typical member of this group, namely, glue (Gr. κόλλα = glue). He found, also, that the substances included in his group of colloids were not capable of passing through a membrane of parchment which offered little or no obstacle to the passage of crystalloids such as sugars and salts. As a first approximation it can be said that the reason why a colloid in solution will not diffuse through a membrane is that its molecules are too large to pass through the spaces between the molecules of the substance of the membrane. This has an important bearing on the significance of the process of digestion, for the great bulk of the soluble constituents of ordinary foods—proteins, starches, for example—are colloidal in nature, and so, therefore, would not pass at all readily by a process of diffusion through the layers of the mucous membrane separating the cavity of the small intestine from the blood vessels. But during the changes that occur in digestion the large molecules of these colloids are broken down, as we have seen, into simpler molecules—amino-acids, sugars—all of which are members of the crystalloid group, and so are characterised by rapid diffusion. From this point of view we may say that the result of digestion is to convert the colloidal constituents of the food into simpler crystalloid products to which animal membranes are permeable.

Of these crystalloids some, at least, are resynthesised to form the colloidal constituents of the cells and tissues of the body—the sugars to form glycogen and the amino-acids to form proteins. But it will be realised from what we have already said on a previous page (50) that the

advantage of this digestive breakdown of the food to the crystalloid condition is not merely that it facilitates absorption of the products from the intestine, but also that it provides an opportunity for the recombination of these products in proportions and arrangements differing widely from those which originally existed in the food. Thus from vegetable starch, animal starch, glycogen, can be formed; from the proteins of mutton, those of man.

It is not merely such naturally occurring substances as proteins and starch that exist in the colloidal solution condition. Colloidal solutions of many metals and their oxides and sulphides were known long enough ago to be investigated by Faraday. Of course these substances do not disperse themselves spontaneously into colloidal solutions, as do starches or proteins, when shaken up with water; their solutions are obtained by causing the required material to be precipitated in exceedingly minute particles, or alternatively in the cases of metals by forming an electric arc between two electrodes of the particular metal desired immersed in pure water. In this second case the metal is probably vaporised in the intense heat of the arc, and the vapour is immediately condensed in minute particles when it comes into contact with the cold water. In this way there are produced myriads of metallic particles fine enough to remain practically permanently in solution, but large and sluggish compared with small active molecules of crystalloids in true solutions. Large enough, in fact, to catch light that falls upon them, and to scatter it sideways. Hence if the colloidal solution is illuminated by rays passing in a horizontal direction, the scattered light will pass up the tube of a microscope, and under suitable magnification each colloidal particle will appear as a bright point. It is by means of such an

arrangement — known as the ultramicroscope — that information regarding the number, size and movements of colloidal particles can be obtained.

There is one important respect in which the colloidal solutions of proteins and starches differ from those of platinum and gold and of other inorganic materials which have been obtained in the colloidal or dispersed condition. This lies in the intimacy of the relationship between the colloidal particles themselves and the water in which they are suspended. For while the particles of metal in a platinum "sol" are more or less independent of the water molecules, so that the system as a whole forms a thin liquid like water itself, the molecules of a dissolved protein seem to be combined in some way with a considerable amount of water, so that the solution is sticky and viscous and readily forms a jelly-like structure or "gel."

Limpid **hydrophobic** (Gr. *ὕδωρ* = water, *φόβος* = fear) colloidal solutions of the type represented by platinum sol are ordinarily referred to as **suspensoids**; the viscous **hydrophilic** (Gr. *φίλια* = love) colloidal solutions such as those of proteins are known as **emulsoids**. Silicic acid (hydrated SiO_2) is one of the few inorganic substances to form a colloidal solution of this type. From the point of view of physiology the emulsoids form by far the more important group because it includes all the colloidal constituents of living matter, and in particular the universally present proteins, and there is little doubt that many of the properties and attributes of living protoplasm, such as its viscosity, its power of imbibing water, its ability to admit into or to exclude from the cell substances that occur in the external medium are largely dependent on the emulsoid properties of these essential constituents. Emulsoid colloids, on account of their affinity for water molecules, are held

relatively much more firmly in solution. They are much less sensitive to, i.e. much less easily precipitated by, salts than are suspensoids. It seems, however, that what happens when a coagulable protein undergoes the change that we have on several former occasions referred to as "denaturation" is that it in some way loses its hold on the water molecules with which it was previously associated as an emulsoid and thus becoming a suspensoid is easily thrown out of solution, often in the form of a coagulum. This is easily illustrated by boiling a solution of albumin in dilute acid or alkali. No obvious change takes place in the solution, but, nevertheless, the protein is altered; it can now be precipitated in the denatured, metaprotein condition by neutralising the solution or half saturating it with ammonium sulphate—neither of which procedures would have affected the original untreated native albumin. And there is reason for believing that when proteins in the body lose in a somewhat similar way their associated water molecules they are virtually dead and are fit merely to serve as the material of such inactive structures as hair and wool and connective tissue fibres. A somewhat similar but reversible change of emulsoid colloids to suspensoids and back again has been described as occurring during the movements of protoplasm as, for example, in the pseudopodium of an amoeba.

A factor of great importance in the maintenance of the stability of all colloidal solutions is the circumstance that their dispersed particles carry an electric charge. This readily manifests itself by the movement of the particles that takes place when an electric current is passed through a colloidal solution. In many cases it is easy to understand how this charge is acquired. For example, in the case of a platinum solution doubtless the particles

send into the water platinum ions, which, being metallic ions, carry a positive charge, and so leave the remaining metallic particle negatively charged. A protein molecule in slightly alkaline solution acts as a weak acid, and therefore tends to send off positively-charged hydrogen ions into the solution. On the other hand, in acid solution a protein acts as a base—resembling in this way the amphoteric amino-acids of which it is composed—and so tends to split off negatively charged hydroxyl ions so that the remaining bulk of the molecule forms a positively charged ion. It is evident that at a certain intermediate reaction a given protein will tend to give off both hydrogen and hydroxyl ions to equal extents; the remaining bulk of the molecule will then possess equal positive and negative charges, that is, it remains neutral. The reaction at which this occurs is said to be the **isoelectric point** of the particular protein (or other amphoteric substance), because at this reaction the protein molecules are not electrically charged, and therefore do not move in an electric field. We see then that either positively or negatively charged colloids may exist, the charge determining the direction in which they tend to move when they are submitted to an electric current. As we have said, the presence of this charge helps to render the colloidal solution stable and to prevent the suspended colloidal particles from coming together and adhering to form larger masses which would separate out of the solution. For the particles, possessing charges of similar sign, repel one another, and so are kept apart. If, however, there be added to a colloidal solution ions carrying a charge of sign opposite to that carried by the colloidal particles, then when such an ion meets a colloidal particle their charges will neutralise each other, at least partially, and if

a sufficient number of these discharging ions are present, all the colloidal particles will lose their charges, and so will adhere together and settle out. Naturally the discharging power of an ion depends on the amount of charge it carries, and for that reason divalent ions have a much more powerful precipitating action than monovalent ions possess, while trivalent ions will act even in minute concentrations on account of the three units of charge which each carries. Of course, a given colloid will be precipitated only by ions that carry charges opposite in sign to its own. This relation of substances in colloidal solution to charged ions is an important consideration, for the chief characteristic of a colloidal solution being its apparent stability, the study of any factor which tends to modify this characteristic property and to precipitate the colloid must necessarily give us further insight into the nature and properties of the colloidal system itself. And as we shall see later, there is little doubt that the powerful actions exerted by ions in living tissues are due to the modifications that they produce in the condition of the colloidal constituents of the cell.

From what we have said it will be easy to understand how it is that proteins are most readily precipitated when they are in the isoelectric condition, for then there is the maximum number of uncharged particles in solution so that aggregation can most readily occur.

Colloidal particles may be discharged and precipitated not only by ions, but also by other colloidal particles, carrying the opposite sign of charge. This fact is applied in the use of colloidal ferric hydroxide ("dialysed iron"), which carries a +ve charge, for the removal of the proteins (which in alkaline solution are -vely charged) from such fluids as blood or milk as a preliminary to the estimation

of their crystalloid constituents. On the other hand the converse case in which one colloid protects another against the precipitating action of ions is also quite common. Faraday knew that a trace of "jelly" would prevent the precipitation of colloidal gold by salt, and the phenomenon is now utilised in medical practice for obtaining an idea of the relative concentrations, in such a material as cerebrospinal fluid, of albumin, which, like gelatin, protects, and globulin, which sensitises, the colloidal gold. The net effect of the fluid is observed by setting up known dilutions of it with standard quantities of sodium chloride solution and gold sol, and from its relative protecting or sensitising action the ratio of albumin to globulin is deduced.

From the examples we have given it will have been abundantly realised that colloidal particles are never, even at the best of times, very firmly in solution, although, owing to continuous bombardment by the water molecules and the repulsive action of their similar charges, the rate at which they tend to separate out under the force of gravity alone may not be noticeable. But when they are exposed to forces up to a million times that of gravity (!), by being rotated at 160,000 revolutions a minute in a turbine-driven ultra-centrifuge, even proteins settle out of solution, and this fact has been taken advantage of for estimating from the rate of settling under given conditions the size of the particles in protein solutions. If we regard these particles as molecules the striking result is obtained that the molecular weights of the native proteins are all multiples of a fundamental unit of approximately 34,500. Thus egg-albumin is found to have a "molecular weight" of 34,500 and hæmoglobin, as was mentioned from other evidence in Chapter XVIII, of about 68,000, i.e. twice the

unit value. The molecular weights of some other proteins appear to run into millions. But it is questionable whether in the case not only of the proteins, but of other biological products of high molecular weight (cellulose, starch, india-rubber) the concept of molecule in the exact chemical sense is strictly applicable; it is possible that a protein colloidal particle consists of an aggregate or **micelle** of similar peptide chains which on separation by some process of ultramicrodissection would still all possess identically the same *chemical* properties as the original aggregate from which they were cut. And this idea is borne out by the result of X-ray diffraction analysis of the crystal structure of these "supermolecular" substances.

Many of the other of the properties of matter in the colloidal state arise from the circumstance that the particles of the dispersed material together present such an enormous area to the liquid in which they are dispersed. In other words, in colloids there are abundant opportunities for the display of surface effects. This will be vividly realised when we mention that if a sphere of material of the size of a tennis ball were to be subdivided into particles of the size ordinarily found in colloidal solutions the total aggregate area of all these particles would amount to twenty times that of the area of a tennis court! In describing the properties of the bile salts we explained briefly the nature of surface energy (p. 128). Now although they possess the property in a very marked degree, the bile salts are not the only substances that have the effect of lowering the energy of a liquid surface. Many other dissolved solids show the same property, although to a lesser extent, and the more of such a substance is present in solution the greater the lowering of the surface tension. Now in all processes

involving energy it is a matter of universal observation that the free energy of any system tends to become a minimum, and that if any change in the system will produce a reduction of the free energy, then that change tends to take place. For example, a mass of matter tends to lose its free energy by falling from an initial position to a point nearer the earth; heat tends to flow from hot to cold bodies so that equality of temperature is established, and the heat energy, although undiminished in amount, is no longer "free," i.e. available for conversion into mechanical work — and so on. If now we consider the case of a solution of a substance whose property it is to lower surface tension, it will be seen that if we start with the dissolved solid uniformly distributed throughout the mass of liquid, we could diminish the free energy of such a system by causing the dissolved solid to leave the main bulk of the solution, and to be accumulated as much as possible in the surface layers, where it produces its lowering of surface tension. As a matter of fact, such a change would occur spontaneously; it is, indeed, found that any substance that reduces surface energy exists in greater concentration in the surface layers of a liquid than in the main bulk of it. The dissolved solid diffuses into the surface layers until the diminution of surface tension it produces is just balanced by its tendency to diffuse back into the main bulk of liquid where its concentration is less.

This process of surface concentration of a dissolved substance is called **adsorption** to indicate that it involves merely an accumulation of the substance at the surface as opposed to *absorption* which involves the swallowing up of one substance into the mass of another. It is not always easy to distinguish this purely physical adsorption of a substance at a surface from its concentration there as

a result of strictly chemical forces, but in this latter case the substance will be concentrated at certain points in the surface determined by the localisation of the chemically reactive groups, while in the case of physical adsorption it will be distributed as uniformly as the molecular structure of matter permits over the whole of the adsorbing surface. Many of the most typical processes of adsorption occur on indifferent surfaces of glass or charcoal which can hardly be held to exert any very powerful chemical attraction on the materials they adsorb.

One of the most striking examples of adsorption is that of potassium permanganate on glass. This salt lowers the surface energy at the surface of contact between glass and water, and therefore tends to become concentrated at such a surface, but the effect is too small to enable one to detect by mere inspection any increased depth of colour at the sides of a glass beaker containing permanganate solution. If, however, we expose the solution to a very extensive area of glass by allowing it to trickle through a tube packed with glass wool then the permanganate is adsorbed to such an extent that the first portions of the liquid to drop from the lower end of the tube will be practically colourless. Then again, the process of adsorption can be readily illustrated by the reduction in concentration that occurs when solutions of such substances as iodine, acetic acid, or acetone are shaken with powdered charcoal. Naturally it is of the greatest interest to determine the relation between the amount of substance adsorbed per unit area of surface and the concentration of it left in equilibrium in the bulk of the solution. By direct chemical analysis of the solution before and after the adsorption has taken place it is found that in many cases the concentration of the adsorbed substance is proportional

to the square, or some near, root of the concentration in the solution. So that if c_a represents the concentration of the solute in the adsorbed layer and c_s the concentration of the substance left in the solution,

$$c_a = k \sqrt[n]{c_s}$$

i.e.
$$c_a = k \cdot c_s^{\frac{1}{n}}$$

where n is a number approximately equal to 2, and k is a constant whose value depends on the particular substance, solvent and surface used.

We have said that this equation has been deduced merely empirically from the observed results of experiments; but by equating the reduction of surface energy (expressed as a first approximation by the reduction of surface tension) produced by the concentration of the solute in the surface to the energy with which the solute tends to diffuse back into the more dilute bulk of the solution it is possible to calculate the concentration of solute in the surface layers in terms of its effect on the surface tension, and from this, if we make certain simplifying assumptions, it is possible to connect up surface tension and concentration, and so to show that in the simpler cases this exponential law of adsorption is what would be expected on theoretical grounds.

When this relation is plotted the curve obtained is a parabola (Fig. 20), from whose shape it is at once evident that the *proportion* of adsorbed to dissolved solute is relatively much greater over the initial rapidly-rising portion of the curve than over its later course, which means that at low concentrations a much greater *fraction* of the total solid will be adsorbed than at high

concentrations. In the course of practical chemical work one often comes across phenomena that illustrate this behaviour. The efficiency of charcoal as an adsorbent for

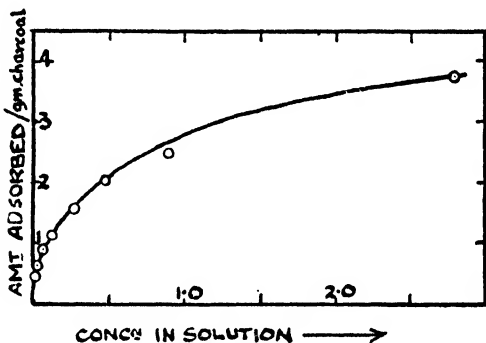


FIG. 20. Adsorption of acetic acid from aqueous solution by charcoal (data from Hedges).

small quantities of highly-coloured impurities in organic preparations is given everyday application. The same property of charcoal can also be utilised for the removal of disaccharides from monosaccharides occurring together in solution. And then again if the student has to wash up his own glassware he will know how difficult it often is to remove by water alone the last traces of a water-soluble dye that has become adsorbed on to the inside of a flask. The dye is usually, however, easily removed by alcohol, which illustrates the circumstance that adsorption does not take place with equal readiness from all solvents and usually not so easily from solutions in alcohol as from those in water. This same principle is being applied very extensively at the present time for the separation and isolation of enzymes, and also of other easily decomposable substances such as vitamins, bacterial toxins and

antibodies. The desired material is adsorbed on to a suitable specific adsorbent—most commonly alumina or some similar mineral substance—and is then recovered from the adsorption complex by washing out, “elution,” with an appropriate salt solution or other solvent whose surface energy the active material does not affect and so from which it would not be adsorbed.

According to the parabolic law expressed by the above equation and by the curve of Fig. 20 no matter how concentrated the bulk of the solution may be made, any further increase in its concentration should be accompanied by *some* increase in the concentration of solute in the adsorbed layer. But, as we said, it is only to the simpler cases that this law applies. In other cases it is found that a surface tends to become “saturated” and, beyond a certain amount, to take up no more of the solute no matter how much its concentration in the bulk of the solution may be increased. It is not easy to decide by inspection whether the data of an adsorption experiment lie on a truly parabolic curve, but if we express our equation in logarithmic form,

$$\log. c_a = \log. k + \frac{1}{n} \log. c_s$$

we see that in cases where this law is obeyed the logarithm of the concentration of adsorbed substance increases in direct proportion to the logarithm of the concentration of dissolved substance, so that these two quantities when plotted should give a straight line, which is much easier to judge of.

On account of the occurrence of adsorption we shall not expect to find anything approaching a uniform distribution of the salts, sugars and other crystalloids present in cell protoplasm, for its colloidal constituents

must present almost unlimited possibilities for the action of surface forces. And further, seeing that it has been shown by direct experiment that an adsorbed material may be displaced from a surface by a still more powerfully surface-active substance, and that many of the drugs (particularly the anæsthetics) that affect living matter do in fact possess powerful surface-activity, we have here a possible explanation of the actions of these drugs in terms of the displacement of cell constituents from the active surfaces at which they are, normally, metabolised.

Our knowledge of the behaviour of substances at surfaces does not however end here. Langmuir has shown that when a drop of a fatty acid is allowed to spread on the surface of water until it only just reduces the surface tension below that for pure water, until, that is, the fatty acid film is only one molecule thick, the area occupied per molecule is the same no matter what the length of the carbon chain of the fatty acid may be. This can only mean that on the surface of the water the fatty acid molecules are all arranged "on end" so that the area occupied by each is determined by the cross-sectional area of the fatty acid chain (which will be the same for all the acids) and not by its length (which will differ from one acid to another). Undoubtedly it will be the "water-soluble" portion of the molecule, namely the carboxyl group, that is attracted down into the water, and the water-insoluble hydrocarbon chain that is uppermost. The same method of investigation has been applied to a large number of substances, so that we possess a considerable amount of information concerning the orientation of such "polar" molecules at interfaces. Now it might almost have been expected that if molecules are thus orientated at interfaces their chemical behaviour

should be somewhat different from that which they exhibit in the bulk of a solution where no such regular arrangement obtains. And such has been proved to be the case. A very striking example taken from recent work is furnished by the behaviour of the dye malachite green at a benzene-water interface. A solution of this dye in 0.3N. hydrochloric acid is colourless, and if a layer of benzene is floated on to the surface of the solution still no appreciable colour can be seen in the interface, but if now the two liquids are shaken together so as to disperse the benzene into fine droplets, and so very much to increase the extent of the benzene/water interface, the whole mixture turns grass green, because, in the interface, the green form of the dye is stable. On allowing the benzene once more to float up as a compact layer on the surface the colour disappears as the conditions for its stability are removed. It is not at all impossible that the active groups of enzymes, and also of such hormones as insulin, are in a similar way stable at the interfaces of their colloidal "carriers"; but are incapable of existence apart from them. From this one example will be realised the extreme probability that future work will ascribe a very important part in the activities of living matter to surface forces operating at the interfaces that, in spite of its tantalising apparent homogeneity, we know it must possess.

The Reaction of Body Fluids

"The heart knoweth his own bitterness."—*The Wisdom of Solomon.*

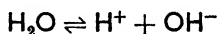
It has been known for a long time that many physiological processes are very profoundly influenced by the reaction of the medium in which they take place. Take,

for example, the heart: if the medium surrounding it, or the fluid it contains, is too alkaline, then it comes to rest usually in a contracted condition. On the other hand, if the fluid surrounding the heart is too acid, then the beats again cease, leaving the musculature this time in a completely relaxed condition. But if the fluid supplied to it is of just the right reaction, the heart will go on beating continuously, alternately relaxing and contracting. The important point to notice is that there is just a very narrow range of reaction over which the heart muscle can function; outside the limits of that narrow range its rhythmic activity cannot be maintained. This is one of the best examples we can choose of the very profound influence of small changes of reaction on the physiological behaviour of a tissue.

From this it will be easy to understand the reason why, in physiological work, it is necessary so frequently to take account of the reaction of the fluids that are being used; for not only the heart-beat, but practically all other physiological processes can occur only in a medium possessing a certain reaction, and are arrested in media that are appreciably more acid or more alkaline. Human life as a whole is like this. Let the blood become ever so slightly acid—not even as acid as distilled water that has absorbed a trace of CO_2 from the atmosphere—and the patient dies in coma; let it become even so faintly alkaline as tap water is, and the subject goes into tetany. Between these two limits we have all our lives to try to steer a middle course.

During the last two decades knowledge of the reaction of solutions has been made very much more definite than it formerly was, because it has now been realised that acid solutions owe their typical properties to the presence

of excess of hydrogen ions, and alkaline solutions owe their alkaline properties to the presence of excess of hydroxyl ions, while a neutral solution is one that contains equal numbers of hydrogen and hydroxyl ions. The typical neutral liquid is water. Pure water is not absolutely free from either hydrogen or hydroxyl ions, but it contains these ions in equal concentrations, for these ions arise by the electrolytic dissociation, or, as we say, the ionisation of a certain number of the water molecules. One can represent the process by an equation as follows:—



Now it is evident that by the ionisation of water there must necessarily be produced equal numbers of hydrogen and hydroxyl ions, simply because when a hydrogen ion has been split off from the water molecule, a hydroxyl ion is all that remains.

Suppose we wish to increase the concentration of hydrogen ions in a solution above the concentration of hydroxyl ions, the simplest plan is to add an acid, for example, hydrochloric acid; when this is dissolved in water its molecules ionise, yielding hydrogen ions and chlorine ions, the hydrogen ions as before being positively charged, and the chlorine ions being negatively charged. These hydrogen ions from the acid are added to the hydrogen ions from the water molecules, giving an increase of hydrogen ions over and above the concentration of hydroxyl ions, which have come entirely from the water molecules. On the other hand, suppose we wish to increase the concentration of hydroxyl ions in a solution; suppose, that is to say, we wish to make the fluid alkaline, then there must be dissolved in it an alkali, such as caustic

soda. This will ionise into sodium ions, which are charged positively, and hydroxyl ions, which are negatively charged. Thus there will be present in a solution of caustic soda the excess of hydroxyl ions derived from the soda itself, together with the hydroxyl ions resulting from the ionisation of the water; there will also be those hydrogen ions—just a very small quantity of them—which have arisen also from the water.

It is very important to realise that every aqueous solution, even the most alkaline, still contains a certain small quantity of hydrogen ions. Hydrogen ions are not confined to solutions of acids. A solution is alkaline because it contains a large excess of hydroxyl ions, and if it be an aqueous solution it necessarily must contain some hydrogen ions because water always ionises to a small extent, giving hydrogen and hydroxyl ions; similarly, in a solution of an acid, even of the strongest acid, there are just a few hydroxyl ions which arise from the water present on account of the tendency of the water molecules to ionise.

An acid solution can never be completely free from hydroxyl ions, nor an alkaline solution free from hydrogen ions. But it must be pointed out that this ionisation of water is a balanced reaction, so that when an excess of any product of that reaction, such, for example, as hydrogen ions—when an excess of these ions is added to the system, then the reaction tends to go in the backward direction, for some of the excess hydrogen ions will combine with some of the hydroxyl ions to form water molecules. Therefore, in an acid solution, containing an excess of hydrogen ions, although there will always be some hydroxyl ions present, there will be a lower concentration of these hydroxyl ions than in pure water. On

the other hand, if an excess of hydroxyl ions be added to this system, then some of these excess hydroxyl ions will combine with some of the hydrogen ions which have been obtained from the water molecules, so that there will be less hydrogen ions in the alkaline solution than there are in pure water. But there will always be *some* hydrogen ions in the alkaline solution.

We can express these relationships much more exactly, for the law of mass action applies to a reversible equilibrium such as ionisation of water. The law of mass action states that the product of the concentrations of all the reacting molecules on one side of the equation bears a constant ratio to the product of the concentrations of the molecules that are formed in the reaction. This is true of all chemical equilibria. If we apply it to our particular case we conclude that the product of the concentration of hydrogen ions and the concentration of hydroxyl ions bears a constant ratio—let us call it k —to the concentration of the water molecules in any system where these three constituents occur together. The concentration of each substance is expressed in the usual way in gram-molecules per litre, so that by the concentration of hydrogen ions is meant the number of gram-molecules of hydrogen ions per litre of the solution. A solution of unit concentration of hydrogen ions—a so-called “normal” solution—will contain one gram-molecule of hydrogen ions per litre. And since the gram-molecular weight of a hydrogen ion is 1, normal concentration of hydrogen ions will be 1 gram per litre. On the other hand, the normal concentration of hydroxyl ions will be 17 grams per litre, for the gram-molecular weight of a hydroxyl ion is 17. ($O + H = 16 + 1$.) If now we write the concentration of hydrogen ions as $[H^+]$,

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the concentration of hydroxyl ions as $[\text{OH}^-]$, and that of the water molecules as $[\text{H}_2\text{O}]$, we can represent the application of the law of mass action to the ionisation of water by the equation

$$[\text{H}^+] \times [\text{OH}^-] = k [\text{H}_2\text{O}]$$

Now we shall see immediately that the actual number of water molecules ionising in any solution is an extremely small fraction of all that are present. This is true even in the case of pure water or a neutral solution, and when acid or alkali is added the ionisation is still further depressed. It is, therefore, true to say that in spite of any changes which may occur in the degree of ionisation, the total number of dissociated water molecules is so small that they may be neglected in comparison with the relatively enormous concentration of undissociated water molecules. In other words, we are justified in overlooking the fact that each change in reaction involves either the formation or the ionisation of a few water molecules, and so can count the concentration of undissociated molecules of water as an unvarying quantity. We will call it c , so that our factor $[\text{H}_2\text{O}]$ is replaceable by c ; then

$$[\text{H}^+] \times [\text{OH}^-] = kc$$

Since both k and c are constant quantities their product is also a constant, so that instead of writing kc we can write one large K to stand for the two constants multiplied together. Then

$$[\text{H}^+] \times [\text{OH}^-] = K$$

This K is called the ionisation constant of water.

About this last equation there are two important things to say. One is that it applies to any system whatever that contains water and hydrogen ions and

hydroxyl ions. This will include pure water itself, and, as we have said, all aqueous solutions. In an acid solution the concentration of hydrogen ions will be large. But the product of the hydrogen ion concentration and the hydroxyl ion concentration is necessarily constant, as we have seen; therefore the hydroxyl ion concentration must be correspondingly small. On the other hand, in an alkaline solution the hydroxyl ion concentration is large. The product of hydroxyl and hydrogen ion concentrations is always the same constant, K , therefore the hydrogen ion concentration in an alkaline solution must be correspondingly small. This is merely a quantitative mathematical statement of what we have already learnt about solutions of hydrochloric acid and caustic soda. In all solutions—acid, alkaline and neutral—if the concentration of hydrogen ions be doubled the concentration of hydroxyl ions becomes halved, for one half of them combine to form water with some of the excess hydrogen ions. The product of their concentrations thus remains constant.

The other important topic connected with this equation is the value of K . In pure water we have already seen that the hydrogen ion concentration is necessarily equal to the hydroxyl ion concentration. That means to say, then, that for pure water $[\text{OH}^-] = [\text{H}^+]$, so that the equation can be written

$$[\text{H}^+]^2 = K$$

So K becomes equal to the square of the concentration of hydrogen (or hydroxyl) ions. This is true only for the particular case of pure water or of a perfectly neutral solution. Now if we have some means whereby we can analyse water, and find out what is the concentration of hydrogen ions in it, then we shall be able to determine this value of K ; and such methods of analysis have been

devised. By physico-chemical methods it is possible to determine the hydrogen ion concentration of pure water, and it is found to be one ten-millionth of normal, that is to say, in a litre of pure water there is only one ten-millionth of a gram of hydrogen ions. It is more convenient to represent this not as a fraction, but as 10^{-7} , which means one ten-millionth, for $10^{-7} = \frac{1}{10^7}$, which is $\frac{1}{10,000,000}$. The hydrogen ion concentration of pure water, or of a neutral solution, is then $10^{-7} \times N$. It therefore follows that our constant K —the ionisation constant for water—is equal to $[10^{-7}]^2$, i.e. 10^{-14} . This means to say that in every aqueous solution the product of the hydrogen ion concentration and the hydroxyl ion concentration is always equal to 10^{-14} .

The hydrogen ion concentration in pure water or in a neutral solution is $10^{-7} \times N$.; in an alkaline fluid such as pancreatic juice it is about $10^{-8} \times N$., while in a fluid as acid as gastric juice (which contains a concentration of about 0.1 N. hydrochloric acid) it reaches at least $10^{-1} \times N$. It will thus be seen that in dealing with hydrogen ions in biological work we have to cover a much wider range of concentrations than is ever used by the chemist in his standard volumetric solutions: for this reason it has been found convenient to express these hydrogen ion concentrations by naming only the index—the power or potency of 10—which is required to express the concentration. An index or power used in this way is termed a pH. Instead, then, of saying that the hydrogen ion concentration in a certain solution is 10^{-7} , it is usual to say that the solution has a pH of 7. It is to be noted that the negative sign of the index is omitted in the pH value. In other words, the pH tells us the negative value of the

power to which 10 must be raised in order to give the actual concentration of hydrogen ions in gram-molecules per litre. Conversely, therefore, a solution of (say) pH 6.5 has a concentration of hydrogen ions of $10^{-6.5}$, i.e. $10^{0.5} \times 10^{-7} = \text{approximately } 3 \times 10^{-7}$ grms. per litre, and so on. But here a little confusion is liable to arise. A solution of hydrogen ion concentration 10^{-7} has a pH of 7, as we have said. Now a solution of hydrogen ion concentration 10^{-6} , that is one-millionth of a gram-molecule per litre, has, of course, 10 times the concentration of hydrogen ions that the first solution of pH 7 possessed, but according to our scheme the pH of the second more acid solution must be written as 6. In other words, then, as the hydrogen ion concentration increases, the pH number diminishes, in such a way that every diminution of unity in the pH means an increase of hydrogen ion concentration to ten times the former value, so that a solution of pH 6 has ten times the hydrogen ion concentration of a solution of pH 7 and a solution of pH 5 has ten times the hydrogen ion concentration of a solution of pH 6.

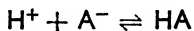
Now we said that a neutral solution has a pH of 7. Solutions with pH's smaller than 7 will be acid solutions, and solutions with pH's greater than 7 will then be alkaline solutions. So that from a slightly different point of view we can say that the larger the pH number, the more alkaline the solution is. We may summarise the whole matter symbolically thus:—

acid	$[H^+] > 10^{-7}$ N. $> [OH^-]$ $pH < 7 < pOH$
neutral	$[H^+] = 10^{-7}$ N. $= [OH^-]$ $pH = 7 = pOH$
alkaline	$[H^+] < 10^{-7}$ N. $< [OH^-]$ $pH > 7 > pOH$

It must always be clearly realised that the pH scale, like the musical scale, is a logarithmic scale, so that pH 6.5 does not represent a concentration of hydrogen ions which is arithmetically half way between those represented by pH 7 and pH 6 respectively, and so on.

Now that we are familiar with the rather special nomenclature that it is convenient to use for expressing the wide range of hydrogen ion concentrations we have to deal with in biological investigations we can go on to consider more exactly and mathematically the ionic equilibria in solutions of weak acids and bases.

Let us consider the case of a weak acid such as acetic, i.e. one that is not completely ionised in solution. In its solution we shall have an equilibrium between the hydrogen ions, the anions of the acid (A^-) and the acid molecules (HA) remaining unionised, thus



Now this equilibrium will be subject to the general law of mass action, so that representing gram-molecular or gram-ionic concentrations in square brackets as before we may write

$$[H^+] \times [A^-] = k [HA]$$

or

$$[H^+] = \frac{k [HA]}{[A^-]} \dots\dots\dots(1)$$

where the constant k is now the ionisation constant of the acid, and this will be universally true no matter how these reacting constituents have been brought together, no matter whether some of the hydrogen ions present have been derived from some other acid in the solution or have all been derived from the HA. But we are expressing

hydrogen ion concentrations in terms of pH, so taking logarithms of both sides we have

$$\log. [H^+] = \log. k + \log. \frac{[HA]}{[A^-]}$$

or, changing the signs throughout

$$\begin{aligned} -\log. [H^+] &= -\log. k - \log. \frac{[HA]}{[A^-]} \\ &= -\log. k + \log. \frac{[A^-]}{[HA]} \end{aligned}$$

i.e.
$$pH = pK + \log. \frac{[A^-]}{[HA]} \dots\dots\dots (2)$$

where pK represents $-\log. k$ on analogy with pH.

Now this is an equation of fundamental importance in connection with a very large number of problems in which acid/base equilibria are concerned. It expresses the way in which the degree of ionisation of the acid is depressed as it finds itself in solutions of progressively increasing hydrogen ion concentration or is increased in more alkaline ones. Conversely it enables us to deduce the pH of the solution if we know the proportion of acid in the ionised form. These relationships will be realised most vividly if we convert equation (2) into a diagram by calculating from it the proportion of ionised to *total* acid, i.e. the ratio $\frac{[A^-]}{[HA] + [A^-]}$ at various pH values and plotting these against one another. We do this because what we are really interested in is not the ratio of ionised to unionised acid, but the ratio of acid ionised (i.e. yielding hydrogen ions) to the total amount of acid we weighed out and dissolved up. The reader will be able for himself to perform the simple mathematical operation for deriving this expression from $\frac{[A^-]}{[HA]}$, the ratio of ionised to unionised

acid, which is what the equation directly gives. He will then obtain the following figure :

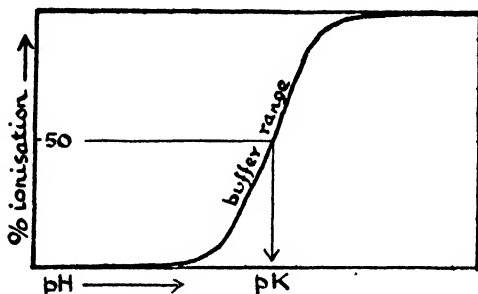


FIG. 21. Ionisation curve of a weak acid.

Now the most obvious way of changing the pH of the acid solution in practice is to add successive quantities of caustic alkali. If we actually do this, measuring the pH of the mixture after each addition of alkali, and then plot these pH values against the amounts of alkali added we obtain the **neutralisation curve** of the acid which, if the acid be a weak one—that is not very much ionised anyway—but forms a well-ionised sodium salt, will be practically identical with the curve we have just given. For under these conditions we can, over the greater part of the range of the curves, neglect the small contribution of anion A^- arising from the ionisation of the acid itself in comparison with the relatively large concentration of this ion arising from the sodium salt, and, further, if the sodium salt is practically completely ionised (as sodium salts even of weak acids usually are) we can take the concentration of anions arising from it as equal to the concentration of sodium salt itself, and this will be the same as the amount of soda added. Under these circumstances we can, in

other words, write $[\text{NaA}]$ instead of $[\text{A}^-]$ and our formula then becomes:

$$\text{pH} = \text{pK} + \log. \frac{[\text{NaA}]}{[\text{HA}]} \dots\dots\dots (3)$$

which tells us that the pH of any solution containing a weak acid and its sodium salt is determined by the *ratio* of these two components present, the mixture being more alkaline the greater the proportion of sodium salt and more acid the greater that of acid. It is as if the sodium salt determined the alkalinity of the solution and the acid the acidity and the ratio of salt to acid the actual prevailing reaction of the mixture.

Now on referring back to Fig. 21 it will be at once evident that the first addition of alkali to the acid produces a large change in the pH, but that further additions produce less and less change until in the mixtures in which about half the acid is neutralised the change produced by a given amount of alkali is a minimum; when, however, most of the acid has been neutralised the further addition of alkali now once more produces a large change of reaction. In other words, the shape of the curve indicates that over the mid-portion of the range the mixture of weak acid and sodium salt shows a considerable degree of stability so far as its hydrogen ion concentration is concerned—alkali can be added to it without making it very much more alkaline and acid could similarly be added without making it very much more acid. Such a mixture is known as a **buffer solution**, because, like the buffer of a railway train or a car, it “eases the shock” of the addition of acid or alkali and allows of only gentle changes of hydrogen ion concentration even when strong acids or bases are added. If a strong mineral acid be added to a mixture of sodium acetate and acetic acid it will decompose a portion of the

sodium acetate and liberate an equivalent amount of acetic acid, which, in the presence of the rest of the sodium acetate, will be but feebly ionised and so will not contribute a great addition to the hydrogen ion concentration of the solution. On the other hand, if a strong base be added to the same mixture it will neutralise some of the acetic acid, but as this was not much ionised in the presence of the sodium acetate no very great loss of hydrogen ions will result.

Now all that we have said so far obviously applies not only to acetic acid but to all weak monobasic acids that form well-ionised sodium salts, so that we may make buffer mixtures with (among many other substances) lactic acid and sodium lactate, carbonic acid and sodium bicarbonate; also with sodium dihydrogen phosphate NaH_2PO_4 (which still contains replaceable hydrogen and so may be regarded as a weak acid) and disodium hydrogen phosphate Na_2HPO_4 (which may be regarded as the corresponding sodium salt). We shall see that the bicarbonate buffer system is of importance in the blood; the phosphate system is the chief means of buffering in the tissues and in urine. The variations of hydrogen ion concentration in all of these mixtures follow exactly the same law as is expressed in Fig. 21, the only difference between them being in their several values of pK which alters the position of the curve on the diagram (and so the pH range over which the buffer action is effective) but not its shape. It is easy to show that in every case the curve comes into such a position that the pH corresponding to the point at which half of the acid is neutralised is numerically equal to the pK of the acid used. For at this half neutralisation point $[\text{HA}] = [\text{A}^-]$ so that $\frac{[\text{A}^-]}{[\text{HA}]} = 1$ and its logarithm therefore is zero.

It is of great importance to distinguish this buffer action from mere neutralisation. If we take a caustic soda solution and add an acid to it we might end up with a mixture that was practically neutral. But in this case a very large change in reaction from say pH 10 to pH 7 would take place. If, however, we take a buffer mixture we could have started with a solution whose pH was less than 8 and after adding the same amount of acid, might finish with a solution whose pH was still greater than 7.

Buffer solutions are of great use in many ways. They find one of their applications in experiments in which it is desired to maintain a constancy of hydrogen ion concentration during the course of some process or reaction, as for example to prevent an acid-producing enzyme from being inhibited or destroyed by the acid products of its own reaction. From what we have said as to the actual pH of a buffer solution being dependent only on the ratio of its more alkaline and more acid constituents it will be evident that within limits the dilution of a buffer solution with a reasonable amount of a more or less neutral solution will leave the pH in the mixture practically unchanged, so that in such experiments it is permissible to assume that if a c.c. or two of such material as an enzyme preparation are added to 10 c.c. of a buffer solution, the pH of the mixture will be the same for all practical purposes as that of the original buffer solution.

Then again buffer mixtures find extensive use as standards of hydrogen ion concentration. At first sight it might seem as if it would be possible to use very dilute solutions of plain acids and alkalis for this purpose, but a moment's reflection will show that in order to have a

solution of pH 6 it would be necessary to have a one-millionth normal solution of a strong acid; or similarly to obtain a pH of 8 a one-millionth normal solution of a caustic alkali. Now although such solutions might if carefully prepared possess the desired hydrogen ion concentrations when first made up, their pH values would be far from stable. The first trace of carbon dioxide absorbed by the soda from the breath during pipetting, or the first trace of alkali dissolved by the acid from the glass of its bottle, would respectively neutralise such a large relative proportion of these constituents that the pH values of their solutions could not be relied on for an hour at a time. In order then to obtain stable solutions that can be stored for months without change of pH, buffer mixtures must be used. The buffer solutions used as standards are those whose constituents can be readily obtained pure and made up in accurately known concentrations. The pH values of the various mixtures must be determined once for all by the absolute electrical method in which the concentration of hydrogen ions is deduced from the E.M.F. set up at a hydrogen electrode immersed in the solution. This has been done now for a large range of buffer mixtures and the results are tabulated in treatises dealing with practical work on this subject, so that by the use of these, solutions of any desired hydrogen ion concentration may be readily made up.

These standard solutions then find very extensive application in the determination of the pH values of other unknown fluids by the use of indicators. In order to explain most easily the theory underlying this process it is convenient to take the view that an indicator is a weak acid whose anion is of a colour different from that of the

unionised molecule. Then when it is placed in solutions of various hydrogen ion concentrations it will be ionised to varying extents according to the general behaviour of weak acids depicted in Fig. 21. In the more acid solutions it will be but little ionised and show the colour of the unionised molecule; over a certain range of hydrogen ion concentrations the degree of ionisation will increase rapidly with relatively small change in the pH value until finally in the more alkaline solutions the acid will be practically all in the form of a well-ionised alkaline salt and so will show the pure colour of its anion. We thus come to understand how it is that there is only a certain range of hydrogen ion concentration over which the colour change of the indicator is sufficiently rapid to make it of practical use in the comparison of pH values. This useful range may be quite remote from the point of true neutrality: indeed, it will in general be different for different indicators, for it will in any given case depend on the strength of the indicator as an acid, i.e. on its pK value, and this will differ from one indicator to another. With these principles in mind the practical mode of use of indicators for the determination of unknown pH values will be clear. First an indicator must be chosen such that it is showing its most sensitive colour change in the fluid under examination. The same amount of this indicator is then added to the unknown and to each of a series of standard buffer solutions covering the range of the indicator. That buffer solution is then sought out in which the tint of the indicator is as nearly as possible the same as in the unknown. The pH of the unknown is then the same as that of this particular standard buffer. In practice, especially if the unknown fluid is such a one as urine that possesses a colour of its

own, this colour matching is carried out in a comparator so arranged that a tube of the unknown fluid can be placed behind the mixture of colourless buffer solution and indicator: the unknown fluid's own colour is thus added optically to that of the indicator in the buffer solution, and a fair comparison can be made with the mixture of indicator and unknown, in which both pigments are in the same tube.

Now while this method of comparison of pH values with those of standard buffer solutions is easy to carry out in a well equipped laboratory where facilities for the preparation and storage of such solutions exist, it is evident that it is not very convenient under the less advantageous conditions met with in remote districts or on board ship. Methods for the same purpose have therefore been devised which, by taking advantage of the theory of indicator action sketched above, dispense with the use of the standard buffer solutions. The essential point in these methods is that indicators of known pK values are used, these values being determined once for all in laboratory experiments. In the method devised by Michaelis, one-colour indicators such as phenolphthalein and nitrophenols, which are colourless in the unionised form, are employed. A known amount of an appropriate indicator of this type is added to the unknown solution: this produces a certain depth of colour determined by the concentration of the coloured anion produced. This concentration is estimated by adding noted quantities of the same indicator solution from a burette to a dilute but not necessarily exactly standardised caustic soda solution, in which the indicator will be completely ionised, until the colour matches that of the indicator in the unknown fluid. The volume of indicator thus required

to be added to the soda will be less than that added to the unknown; it will be a measure of the concentration of the coloured anion in the unknown mixture. The total amount of indicator solution added to the unknown being known, the difference between this and the amount known to be ionised gives the concentration of unionised indicator. Knowing then the value of pK for the particular indicator we know all the terms necessary for the calculation of the pH of the unknown according to equation (2). More recently Gillespie has worked out a somewhat similar method in which the more usual two-colour indicators are employed. In principle what he does is to compare the colour given by the addition to the fluid under test of a known concentration of a suitable indicator with optical mixtures of the colours produced by distributing in various proportions the same total amount of indicator between two tubes, one containing dilute acid, and the other dilute alkali and placed one behind the other in a comparator. It is evident that the ratio in which the indicator must be divided between the alkali and the acid respectively, in order that the optically mixed colours shall give a match with the unknown + indicator will be the same as the ratio of the concentrations of indicator anion and unionised indicator in the unknown mixture. In other words the last term in our well-used equation (2) becomes known and we have simply to add its value to that of the pK for the particular indicator used in order to obtain the pH of the unknown fluid.

So much then for the elementary theory of the ionisation of weak acids and its applications. If in exactly the same way we consider the ionisation of weak bases we arrive at exactly corresponding conclusions and equations, the only difference being that of course the base is freely

ionised in acid solutions but not in alkaline so that its ionisation curve forms a kind of converse to that of an acid, thus:—

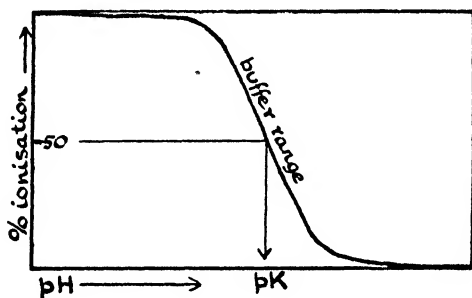


FIG. 22. Ionisation curve of a weak base.

In practice this ionisation of a weak base does not find such frequent application as that for a weak acid; nevertheless there are a few indicators (methyl red, for instance) that are basic and not acidic in character and so ionise according to the curve just given. We need this curve, however, in the consideration of the ionisation of amphoteric substances to which we shall now pass.

The study of amphoteric substances has particular significance in physiology and biochemistry because the amino-acids and with them the proteins, by virtue of possessing both acid (-COOH) and basic (-NH_2) groups are of this type. As we already mentioned as far back as p. 22, an amphoteric substance is one that in acid solution acts as a base, and so on addition to an acid solution it diminishes its hydrogen ion concentration, while in alkaline solution it acts as an acid and diminishes the alkalinity of the solution to which it is added. We may regard such an amphoteric electrolyte (**ampholyte**) as following the ionisation curve of an acid when in alkaline solution,

and then at a certain hydrogen ion concentration suddenly changing over and following the ionisation curve of a base. The actual ionisation curve of the ampholyte will thus come to consist of a part of each of the two ionisation curves thus:—

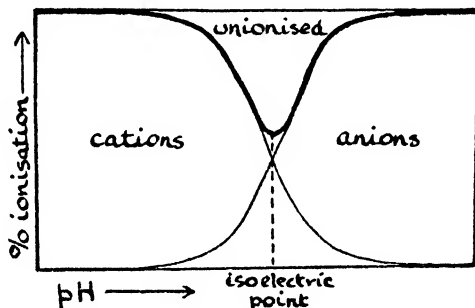


FIG. 23. Ionisation curve of an ampholyte (dark line) made up of parts of the ionisation curves of its equivalent acid and base (light lines).

Of course along one branch of this curve the ampholyte will be forming anions and along the other branch cations, but the great point we wish to emphasise at the moment is that there is a definite hydrogen ion concentration at which the amount ionised in any form, i.e. the amount carrying a charge of either sign, is a minimum. In other words, the residue of ampholyte that is in the form of uncharged molecules is at this point a maximum. The particular pH value at which this occurs is called the **isoelectric point** of the ampholyte. Seeing that the two branches of the ionisation curve are symmetrical about this point it follows that of such portion of the ampholyte as is ionised at the isoelectric point equal amounts will be in the form of anions and cations, so

that the total effective charge associated with the whole of the ampholyte will be nil. Or, to express it differently, at the isoelectric point the ampholyte molecules give off H^+ and OH^- ions in equal numbers leaving single ampholyte ions carrying equal numbers of +ve and -ve charges whose total effective charge will therefore be nil. Such ions are called Zwitterions (German, *Zwitter* = hermaphrodite), and according to modern theory many ampholytes at their isoelectric points are entirely in this form. The effect of acid is then to depress the acid ionisation leaving the ampholyte as +vely charged cations and the effect of alkali is to depress the basic ionisation leaving -vely charged anions. It is evident that the isoelectric point will not necessarily occur at the neutral point, for its position will depend on the positions of the acid and basic ionisation curves and these will depend respectively on the values of the acidic and basic ionisation constants as we have seen. Thus it comes about that we know ampholytes whose isoelectric points are on the acid side and others whose isoelectric points are on the alkaline side of neutrality. Further, the relative positions of the two ionisation curves will determine whether, as in the case we have illustrated in Fig. 23, only a portion of the ampholyte shall be unionised at the isoelectric point, or whether the whole of the ampholyte shall be in the unionised form, or whether again there shall be a wide range of hydrogen ion concentrations over which there will be no appreciable preponderance of +ve or -ve ions present at all.

Now the great importance of this theory of the ionisation of ampholytes lies in its application to the proteins. Proteins, on account of the size of their molecules, form colloidal solutions, and one factor that keeps the protein

securely in solution is the presence of electric charges of similar sign on its colloidal particles, for the mutual repulsion of these similarly charged particles prevents them from coming together to form large aggregates, i.e. from precipitating out. When, however, a solution of a protein is brought to the pH corresponding to its isoelectric point the stabilising effect of the electric charge is removed so that at this point the protein would be expected to show a minimum of security in solution. In the case of some proteins this shows itself as a complete insolubility at the isoelectric point. This for example is the case with the metaproteins into which albumins and globulins are converted on boiling; they are precipitated at their isoelectric point (pH 5.4, the turning point of chlorphenol red) but are soluble in more acid or more alkaline solutions. It is therefore necessary to bring to this pH 5.4 any solution from which albumins or globulins are to be removed by heat coagulation. A similar case is furnished by casein, except that here there is a wide isoelectric zone so that casein, whose isoelectric point is at pH 4.7, and which is soluble enough in alkaline solutions, is precipitated as soon as its solution is made appreciably acid (0.3 per cent. acetic acid or less) and is not dissolved again until the solution is made strongly acid with either 30 per cent. acetic or mineral acid. The region of comparative insolubility thus covers a somewhat wide range of hydrogen ion concentrations. In the cases of other proteins no precipitation takes place at the isoelectric point, but nevertheless the protein is not so securely in solution at this point for it is more easily thrown down by precipitants at this pH. Native (unboiled) albumin, for example, is not precipitated when its solution is brought to the

isoelectric point, but at this point it requires a smaller concentration of ammonium sulphate for its precipitation than in more acid or alkaline solutions. And similarly gelatin is soluble over the whole range of hydrogen ion concentrations but is precipitated more easily by alcohol at its isoelectric pH than at any other.

Naturally it is not merely the solubility that is affected by the charge on the protein particles, so that the isoelectric point comes to be a singular point with regard to a large number of the properties of protein solutions. This is well illustrated by the facts that at the isoelectric point gelatin shows a minimum tendency to swell by absorption of water (because there is the minimum concentration of indiffusible gelatin ions to set up differences of osmotic pressure) and a minimum of viscosity (minimum concentration of charged ions to attract the water molecules and so hinder free movement); but a maximum tendency to set up opaque jellies (gels) (minimum concentration of mutually repulsive ions, hence maximum facility of aggregation into the close network of protein particles to which the characteristic flexibility, elasticity and rigidity of a gel are due). But we cannot do more than just mention these interesting and important phenomena here. Seeing, however, that the properties of proteins are so closely dependent on their degree of ionisation, and that this is determined by the hydrogen ion concentration of the solution in which they find themselves, it is not surprising to find that living tissues, whose essential constituents are proteins, should show a similar exquisite sensitiveness to small changes of hydrogen ion concentration in their environment. In general the tissue fluids seem to be slightly more alkaline than the isoelectric points of their

constituent proteins so that it would seem as if for the manifestation of life phenomena the tissue proteins must be preponderatingly ionised as acids, i.e. as anions, and so carry a negative charge.

This theory of protein ionisation has found another application in the study of enzymes. Many enzymes seem to be amphoteric substances ionising in solutions of various hydrogen ion concentrations in the way we have been describing. Now it would appear that the anions, cations and unchanged molecules of the enzyme have very different degrees of activity in promoting its characteristic reaction, and this gives us an explanation of, at all events some cases where, as we have seen (p 271), the enzyme activity is very much affected by the prevailing hydrogen ion concentration. In the case of pepsin for example, it seems that it is the cation that is particularly active, hence pepsin is most active in acid solution; with trypsin the active agent seems to be the anion, which, of course, is most abundant in alkaline solutions, while in the case of invertase it would seem to be the unionised molecule present in more nearly neutral solution that is the active constituent. This idea gives not merely a qualitative but a tolerably accurate quantitative explanation of the dependence of enzyme activity on hydrogen ion concentration; but that it does not cover all the involved relations between enzymes and hydrogen ions should occasion no surprise.

We will conclude this rapid survey of the importance of hydrogen ions in biological processes with a brief notice of the acid-base equilibrium in the blood. From what we have said it will be realised that the maintenance of a practically constant and appropriate hydrogen ion concentration in the fluid that circulates to every tissue

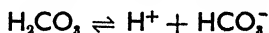
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is a process of prime importance to the body, but it will also be evident that this constancy of hydrogen ion concentration cannot be achieved without very special regulatory mechanisms inasmuch as the main metabolic processes of the body consist of oxidations and the products of oxidation—carbonic acid for example—are acids. From this point of view, in spite of the existence of oxygen-free acids and non-acidic oxides, oxygen is not so inappropriately named the “acid producer.” Acids are always difficult to transport and the problem that the body has to solve is the transportation to the lungs of all the carbonic acid its tissues produce, without any appreciable change in the normal reaction of the blood. Under normal conditions the blood is slightly alkaline; its pH is 7·4 or thereabouts, and the imperative need to which we have already referred of preventing measurable departures from this value may now be expressed numerically by saying that a fall of only about 0·4 (to pH 7·0) leads to coma while a rise of only the same amount (to pH 7·8) produces tetany.

The reader will be easily able to show that these two extremes do not differ from each other by as much as a millionth of a gram of hydrogen ions per litre of blood! Now for the achievement of this amazing accuracy of adjustment the body possesses several mechanisms. The smallest increase in the hydrogen ion concentration of the blood leads to increased breathing, and the consequent more effective washing out of the carbon dioxide from the blood leads to a return of the blood reaction towards the normal value. From this point of view the respiratory centre may be regarded as an organ for maintaining normal the hydrogen ion concentration of the blood. Then again, the kidney excretes a more or a less acid urine

according as the hydrogen ion concentration of the blood tends to rise or fall. But these responses would be of little avail did not the blood itself possess a very high buffering power.

We mentioned on p. 346 that carbonic acid taken up by the blood from the tissues enters into competition with hæmoglobin (which, being on the alkaline side of its isoelectric point, is also functioning as a feeble acid) for the available base of the blood. The result is that a considerable proportion of the carbonic acid is converted into alkaline bicarbonate and only a small fraction remains free. This free carbonic acid tends to ionise into hydrogen and bicarbonate ions thus:—



but this ionisation is very much depressed by the abundance of bicarbonate ions arising from the well-ionised alkaline bicarbonate. If for convenience we disregard the fact that the alkali of the blood is partly potash and represent it all as soda we see that we have here another case of the buffer effect of a mixture of a weak acid with its sodium salt, so that our old equation (3) will again apply. Expressing it in terms of carbonic acid and sodium bicarbonate we have

$$\text{pH} = \text{pK} + \log. \frac{[\text{NaHCO}_3]}{[\text{H}_2\text{CO}_3]} \dots\dots\dots(4)$$

The extent to which the bicarbonate present does actually depress the ionisation of the carbonic acid may well be realised when we point out that if we expose pure water to a partial pressure of 40 mm. Hg of carbon dioxide (i.e. normal alveolar tension) the pH of the resulting solution of carbonic acid will be about 4.7. If

on the other hand we treat a sample of blood in the same way we find that its pH is about 7.4—its hydrogen ion concentration in other words is about $\frac{1}{500}$ of what it would have been were it not for its buffers.

Of course if some stronger acid than carbonic gains access to the blood (lactic acid during exercise, β -hydroxybutyric acid during diabetes) this stronger acid will decompose a portion of the bicarbonate and liberate an equivalent amount of the much weaker carbonic acid. This will inevitably reduce the value of the ratio $\frac{\text{bicarbonate}}{\text{carbonic acid}}$ in the blood, and so, as is easily seen ratio equation (4), will diminish the pH value of the blood, but to a very much smaller extent than if the invading acid had not met this buffer action of the bicarbonate.

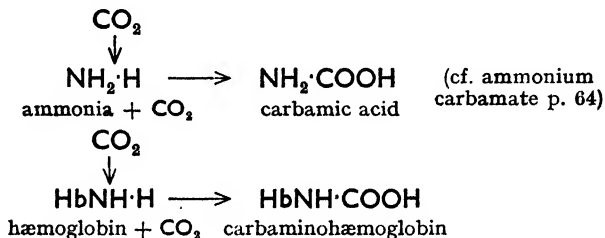
Throughout this account we have more or less definitely implied that the combined carbon dioxide of the blood is entirely in the form of sodium (or potassium) bicarbonate and until recently this was held to be true. The chief support for this view was the observation that equation (4) applies to blood—that is if we analyse a sample of blood to determine the amounts of free carbonic acid and combined carbonic acid it contains and, assuming that the combined carbonic acid is in the form of sodium bicarbonate, substitute these values in the equation, the pH so calculated agrees very nearly with that actually observed by direct measurement. In this calculation the value used for pK is of course that which applies to a plain aqueous solution of carbonic acid. But it has recently been found that if the same argument is applied to solutions of sodium bicarbonate containing considerable concentrations of hæmoglobin (as we have them in the red blood corpuscles) the value that must be assigned to

pK in order to bring the calculated and observed values of the pH of the mixture into agreement is appreciably lower than that characteristic of carbonic acid alone: in other words that the value of the ratio $\frac{\text{bicarbonate}}{\text{carbonic acid}}$ is too large to fit the equation. This has been held to indicate that in the presence of hæmoglobin the *effective* concentration, or, as the physical chemists call it, the "activity" of the bicarbonate ion in the solution is reduced below the concentration actually present, or alternatively that part of the combined carbonic acid present is not in the form of bicarbonate at all, but is combined in some way directly with the hæmoglobin. The way in which this conclusion has been arrived at is interesting. It started with the discovery that when blood is exposed to a vacuum part of its carbon dioxide escapes almost instantaneously, but the remainder comes off much more slowly. This suggested that the carbon dioxide was combined in two different ways. Next it was discovered that in the blood there exists an enzyme that enormously accelerates the apparently simple reversible decomposition of H_2CO_3 into CO_2 and H_2O . This **carbonic anhydrase** as it is called is evidently concerned whenever carbon dioxide is expelled from sodium bicarbonate in the blood because the HCO_3^- ions must first combine with H^+ ions to form H_2CO_3 molecules which then break down—slowly in the absence of the enzyme, or rapidly in its presence—to form CO_2 and H_2O thus:



Now this enzyme can be poisoned by adding cyanide to the blood: when this is done it is found that most of the carbon dioxide is given up only slowly to a vacuum (this "slow" carbon dioxide must therefore have been in the

form of bicarbonate, which decomposes only slowly without the enzyme), but there remains a residue of carbon dioxide that is evolved quickly in spite of the cyanide. This "quick" carbon dioxide would seem to be in a non-bicarbonate form and the suggestion is that it is combined with an NH_2 group in the globin of hæmoglobin to form a carbamino compound, i.e. a substitution product of carbamic acid $\text{NH}_2\cdot\text{COOH}$ thus:



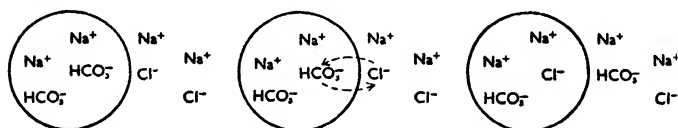
The amount of this **carbamino-hæmoglobin** is small compared with the total carbon dioxide *contained* in the blood, but it is large enough to form a considerable fraction of the amount of carbon dioxide the blood *transports* from tissues to lungs, so that it may play a not unimportant rôle in the body.

There is, however, a further complication in this question of the transport of carbon dioxide by the blood, which arises from the circumstance that the hæmoglobin is confined to the red corpuscles, and so can influence only indirectly the ionic composition of the plasma. It was for a long time difficult to understand the fact that the concentration of chlorine ions is always about twice as great in the plasma as in the corpuscles although chlorine ions would seem to be readily diffusible through cell membranes (p. 359), and the mystery deepened when it was discovered by Hamburger that when carbon dioxide

is removed from blood some of the chlorine ions "shift" from the corpuscles into the plasma—that is they pass from a place where their concentration is lower to one where it is already higher, and therefore quite against the direction in which they would be expected to diffuse. We may emphasise this by saying that the chlorine ions are observed to run uphill—up a slope of chlorine ion concentration—and not down as they might be expected to do. But now that we realise that sodium and potassium ions cannot pass through the membranes of the corpuscles and that chlorine and bicarbonate ions, although themselves potentially diffusible, cannot diffuse freely unless they can take their oppositely charged partners—sodium or potassium ions—with them (this is explained on p. 359) we see that the equilibrium concentrations of the various ions on the two sides of the membrane must be very different from those that would be expected if all the substances concerned were freely diffusible. The influence of a non-diffusible ion on the distribution of other diffusible ones was first worked out by Donnan, and the type of equilibrium attained is universally known as the Donnan equilibrium. In the case of the chloride shift in the blood, what seems to take place is that the carbonic acid diffuses into the corpuscles and there reacts as we have said with the sodium or potassium salt of hæmoglobin, forming an alkaline bicarbonate: the negatively charged bicarbonate ions naturally tend to diffuse out from the corpuscles, where their concentration is initially high, into the plasma (where their concentration is, to begin with, lower) leaving the indiffusible positively charged sodium and potassium ions behind. Such a diffusion could not however go on to any appreciable extent of itself, for it would disturb the condition of electric neutrality and set up an electrostatic field

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between corpuscles and plasma in such a direction as to oppose the further outward diffusion of the bicarbonate ions. But this same electric field causes the movement of negatively charged chlorine ions of the plasma in the opposite direction, i.e. into the corpuscles and this in turn restores the electric neutrality and allows further diffusion of the bicarbonate ions to occur. In this way it comes about that a considerable portion of the bicarbonate ions first formed in the corpuscles passes out into the plasma in exchange for chlorine ions that pass in. So far as the plasma is concerned, then, it starts by possessing sodium chloride and comes to possess, as a result of the addition of carbon dioxide to the blood, sodium bicarbonate, the net result being the same as if the sodium chloride had been to a large extent directly decomposed by the weak carbonic acid under body conditions—a possibility that a chemist would hardly admit. The following diagrammatic representations may serve to make the whole process clear:



CO_2 diffuses through the plasma into the corpuscles and there forms NaHCO_3 by robbing the hæmoglobin of sodium according to the equation $\text{NaHb} + \text{H}_2\text{CO}_3 \rightleftharpoons \text{HHb} + \text{Na}^+ + \text{HCO}_3^-$

the HCO_3^- tends to diffuse out into the plasma and does so by interchange with Cl^- ions

the plasma has now gained HCO_3^- ions and the corpuscles Cl^- ions.

Of course when carbon dioxide is removed from the blood the reverse changes take place. It should be mentioned in conclusion that in actual fact, owing to such secondary

circumstances as the ionisation of the hæmoglobin itself and the passage of a certain amount of water from plasma to corpuscles on addition of carbon dioxide, the numbers of chlorine and bicarbonate ions interchanging do not correspond quite so closely as might have been expected from our simple explanation, but it would take us into too great detail if we entered further into the question here.

The Functional Importance of Electrolytes

“Senza sali nei liquidi periprotoplasmatici non è possibile la vita.”
—*Bottazzi.*

In the preceding section we showed how hydrogen ions exert an influence of such primary importance in determining the degree of ionisation of proteins and so affecting their behaviour in living matter that hydrogen ion concentration now constitutes a fundamental factor in all biological work. But potent as hydrogen ions are in this respect it must not be supposed that they are alone in determining the charge on proteins and thereby influencing the activities of tissues. Proteins combine with other ions by adsorption, and their properties are considerably modified as a result, so that it is not surprising to find that life processes are dependent in a very special manner on the other ions, and particularly on the metallic ions of sodium, calcium and potassium present in the fluid bathing the tissues.

This fundamental discovery was made by Sidney Ringer in the early 1880's during a series of researches, the object of which was to determine the part played by the various constituents of blood in the maintenance of the rhythmic contraction of the ventricle. By perfusing the frog's heart with solutions of various compositions he was able

easily to show that while the proteins and other organic constituents of the blood could be omitted without affecting the activity of the heart musculature to any appreciable extent, any change of the amount of inorganic salts from the concentrations in which they occur normally in the blood led to marked abnormalities in the behaviour of the heart. For example, the complete withdrawal of all dissolved salts by perfusion with distilled water at once

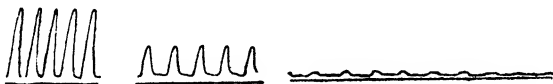


FIG. 24. A copy of Ringer's tracing showing the inability of a solution of pure sodium chloride to maintain the beats of the frog's ventricle.

The first beats were obtained 8 minutes after replacing the blood by 0.75 per cent. sodium chloride solution.

The second series of beats was obtained after a further interval of 6 minutes.

The third series was obtained 4 minutes later than the second.

brought the heart to rest, and destroyed the excitability of its musculature. Distilled water is therefore a poison to tissues. An isotonic solution of sodium chloride is little better than distilled water for the maintenance of the heart-beat, for when it is perfused the heart-beat at once begins to grow feeble, and soon ceases altogether (Fig. 24); the saline solution is, however, somewhat more capable of preserving the irritability of the heart muscle, so that rhythmic beats once more return when a more suitable medium than plain saline is provided.

One of the most abundant inorganic constituents of the blood—apart from sodium—is calcium, and Ringer investigated the effect of adding small quantities of calcium chloride to the saline solution which he passed through the heart. As a matter of fact, he first observed

the effect of calcium accidentally on an occasion when his saline solution had been made up erroneously with tap water instead of with distilled. He found that in a saline solution to which calcium had been added, the rhythmic beats of the heart continued for a time, but that gradually the relaxation of the heart became more and more delayed, until complete relaxation failed to occur before the succeeding systole. This tendency continued until the heart

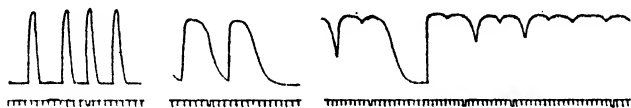


FIG. 25. A tracing (from Ringer) to show the effect of calcium salts in favouring the contracted condition of the ventricular muscle.

The first beats were obtained while the heart was perfused with blood.

The later groups of beats were obtained after the replacement of the blood by sodium chloride solution containing a little calcium.

remained in a permanently contracted condition, and was incapable of relaxing at all (Fig. 25). Calcium, then, favours the contracted condition of the muscle at the expense of the relaxation.

Ringer next tried the effect of a saline solution containing a little potassium chloride, as this metal forms an important constituent of the normal blood. With such a mixture he found that the heart gradually came to rest in a completely relaxed condition. Having thus discovered the opposite tendencies produced by these two metals he further investigated the effect of adding them both to the perfusion fluid. He then found that if the calcium were present in relatively large amount there was the characteristic tendency towards a permanent contraction of the

heart, while if it was the potassium that preponderated, stoppage in a relaxed condition resulted. But if a certain balance between the amounts of the two elements was hit upon, then the heart remained neither permanently contracted nor permanently relaxed, but contracted and relaxed alternately for a period of several hours, just as does a heart supplied with blood (Fig. 26). Indeed, this solution, containing, in addition to a basis of sodium chloride, small quantities of the chlorides of calcium and potassium, provides a practically normal environment not only for heart muscle, but for living tissues in general,

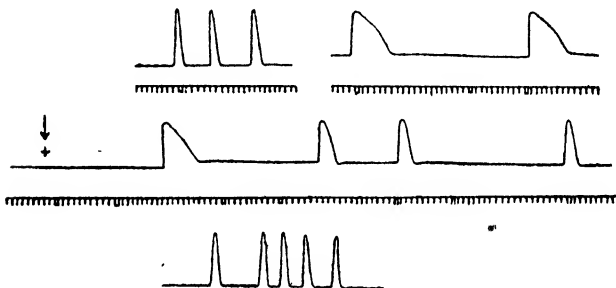


FIG. 26. The antagonism between calcium and potassium (Ringer).

The first recorded beats were obtained with the heart perfused with blood.

The second series was obtained with the heart perfused with a solution of sodium chloride containing calcium. The prolongation of the systole, which is typical of the action of calcium, is again seen.

At the arrow a small quantity of potassium chloride was added to the perfusion fluid. The effects of the calcium are antagonised and the beats become normal.

The last portion of the tracing was obtained 10 minutes after the addition of the potassium chloride.

and is the fluid which is so extensively used in the laboratory under the name **Ringer's solution**, for the preservation of normal conditions in tissues and organs whose behaviour is being studied. Often a little sodium

bicarbonate is added to the mixture in order to preserve a slight alkalinity, and so still further to imitate the conditions existing in the living body. From the remarks we have already made on the subject of the importance of the reaction of body fluids the student will readily appreciate the advantage of this addition.

Ringer was well aware that the effects he discovered were not produced only by the chlorides of potassium and calcium, but were common to all salts of these metals. At the time when he carried out his experiments, the theory of ionic dissociation had not been put forward. We see now that the effects are given not by the salts themselves, but by the positively charged calcium or potassium ions which are split off when the salts undergo electrolytic dissociation or ionisation in solution.

For the proper functioning of the heart, therefore, it is necessary that it should be bathed in a salt solution whose chief constituent is sodium chloride, but which must also contain calcium and potassium ions, and these in such proportions that their antagonistic tendencies are suitably balanced.

And what has been found to be true in the case of the heart has been found also to apply to many other tissues and organs. For the continued lashing of cilia, for the development of the eggs of marine organisms, and for the continued well-being of the animals that arise from them, the same balance of sodium, calcium and potassium ions is essential. And this is so whether we are considering the external environment of an aquatic form or the internal environment furnished by the circulating blood of a land-dwelling beast. Disturb this ionic equilibrium but slightly, and abnormal and harmful effects at once manifest themselves. The delicate organism

dies; the heart ceases to beat, and voluntary muscles which ordinarily remain quite quiescent except during moments of work, now show ceaseless unco-ordinated twitchings by which their energy is frittered away. This last fact is easily demonstrated by placing an excised muscle of the frog in pure sodium chloride solution. It soon commences to show small spontaneous twitchings, which gradually increase in vigour until the muscle begins to grow exhausted, and the movements die away. The addition of calcium salts tends to diminish this activity by reducing the irritability of the muscle; potassium salts increase the vigour of the twitches, and then finally poison the muscle and kill it. But in just the correctly balanced solution of all three salts the muscle will remain at rest, but nevertheless alive and normally irritable, for days at a time. As Loeb said: "We owe it to the calcium and magnesium salts of our blood that our skeletal muscles do not contract rhythmically like our heart."

The student who has followed our discussions up to this point will now be in a position to realise the chief requirements of a fluid which is to be perfused through organs in order to maintain them in as normal a condition as possible when they are out of the body. It must possess a suitable osmotic pressure, so that it shall not damage the tissues by changing their water content; it must be of appropriate, usually slightly alkaline, reaction in order that the excess of the powerful hydrogen ions shall not lead to abnormal changes; and lastly, as we have seen, it must contain such metallic ions as sodium, calcium and potassium in normally balanced proportions.

But Ringer went yet further with the analysis of these

effects of electrolytes. For he made the interesting and important observation that the calcium of his perfusion fluid could be replaced by an equivalent quantity of strontium without destroying the efficiency of the salt mixture in maintaining the heart beat. It is evident therefore that it is not calcium as such which is necessary to the tissue, but that it is the effect of some property common to the calcium and strontium ions that is required for the maintenance of normal activity. Barium was found to be a much less suitable substitute for calcium than strontium—a circumstance not to be wondered at when we remember that of this group of closely related metals barium shows less general chemical resemblance to calcium than does strontium.

In a similar kind of way it is found possible to replace the sodium basis of the perfusion fluid with lithium and its potassium by the related rare element rubidium, and to a less extent by caesium.

Now it is this possibility of replacement of one ion by another which gives us our clearest hint as to the way in which these ions exert their activities in living tissues. For not only do we find that they can be arranged in series showing a regular gradation of general chemical properties, and a corresponding gradation of physiological action, but we also find that similar series will express the several powers which the ions possess of affecting non-living colloidal systems by causing precipitation and such-like changes.

Let us quote an example. It has been found that the cilia of the frog's oesophagus soon became damaged and remain motionless when placed in a pure sodium chloride solution. They thus show a behaviour similar to that of the frog's heart. In a solution of potassium chloride

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they are preserved for a longer period, but even so they eventually die. On the other hand, in a solution of a lithium salt the cilia remain active for an even shorter time than in pure sodium chloride. If in a similar way we examine the rarer alkali metals, rubidium and cæsium, we find that the several ions sort themselves into a series thus:



in which they are arranged in the decreasing order of the length of time for which they will maintain the ciliary movement. Potassium is therefore the least, and lithium the most, poisonous ion.

If now we examine the relative effects of these same ions in the precipitation of colloidal proteins—neutral egg-white, for example—we find that the order of their powers to bring about precipitation (as measured by the smallest concentration necessary to produce a visible effect) is found to be



where lithium produces the least, and potassium the most, precipitation.

We thus see that both in the case of the dead egg-white and of the living ciliated cells the ions come to be arranged in the same order.

From facts like these we conclude that the ions that are indispensable for the maintenance of the normal activities of living tissues are so essential in virtue of the effects they produce on the colloidal protein and other constituents of living matter. And since it is the electric charge carried by an ion which is foremost in producing changes in the colloidal systems, it is only to be expected that the more specific chemical characters of a particular

ion should play a secondary part in its action on living matter. When we find that a strontium ion can play the part of a calcium ion in providing a suitable medium in which the heart can beat we naturally conclude that it is the property common to both—namely, the possession of a double positive charge—which is the chief factor at work in each case; and when we find that the effect of a certain number of hydrogen ions can be imitated exactly by trivalent ions in much smaller concentrations—for now each such ion carries three charges instead of one—when we realise from this for how little the actual chemical individuality of the particular ion counts—we are strengthened in our conclusion.

But when asked how these ions act, we cannot at present make more than the general statement that they exert their physiological action by bringing about changes or maintaining certain conditions in the colloids of the tissues that are submitted to their influence. We are still a long way from explaining the behaviour of living matter in terms of biochemical reactions, of processes of adsorption or the actions of ions on colloids. We have briefly outlined a few of these topics here, but at present they appear as isolated fields of knowledge awaiting the distant time when physiology, biochemistry and biophysics will be no longer separated domains of intellectual endeavour, but will have been synthesised into the unified structure of a single comprehensive science of life.

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